Paul L. H. McSweeney
Patrick F. Fox *Editors*

Advanced Dairy Chemistry

Volume 1A
Proteins: Basic Aspects

4th Edition

Springer

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Volume 1A: Proteins: Basic Aspects, 4th Edition

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Preface to the Fourth Edition

Advanced Dairy Chemistry-1A: Proteins: Basic Aspects is the first volume of the fourth edition of the series on advanced topics in dairy chemistry, which started in 1982 with the publication of *Developments in Dairy Chemistry*. The second and third editions of this work were published in 1992 and 2003, respectively. This series of volumes is an authoritative treatise on dairy chemistry. Like the earlier series, this work is intended for academics, researchers at universities and industry and senior students; each chapter is referenced extensively.

The chemistry and physico-chemical properties of milk proteins are perhaps the largest and most rapidly evolving area in dairy chemistry, and it has proved impossible to cover this topic at the desired depth in one volume. Hence, coverage of dairy proteins in the fourth edition of *Advanced Dairy Chemistry* will be split between basic (this volume) and applied aspects (Volume 1B, forthcoming). All chapters in the third edition on basic aspects of dairy proteins have been retained but have been revised and expanded. The chapters on the chemistry of the caseins (Chap. 4), genetic polymorphism (Chap. 15) and nutritional aspects of milk proteins (Chap. 16) have been revised by new authors, and new chapters have been included on the evolution of the mammary gland (Chap. 1) and on minor proteins and growth factors in milk (Chap. 11).

We wish to thank sincerely the 37 contributors (from 9 countries) of the 16 chapters of this volume, whose co-operation made our task as editors a pleasure. We wish to acknowledge the assistance given by our editor at Springer Science + Business Media, New York, Ms Susan Safren and Ms Rita Beck, assistant editor at Springer, for help in preparing the manuscript.

Cork, Ireland

Paul L.H. McSweeney
Patrick F. Fox

Preface to the Third Edition

Advanced Dairy Chemistry—1: Proteins is the first volume of the third edition of the series on advanced topics in Dairy Chemistry, which started in 1982 with the publication of *Developments in Dairy Chemistry*. This series of volumes is intended to be a coordinated and authoritative treatise on Dairy Chemistry. In the decade since the second edition of this volume was published (1992), there have been considerable advances in the study of milk proteins, which are reflected in changes to this book.

All topics included in the second edition are retained in the current edition, which has been updated and considerably expanded from 18 to 29 chapters. Owing to its size, the book is divided into two parts; Part A (Chapters 1–11) describes the more basic aspects of milk proteins while Part B (Chapters 12–29) reviews the more applied aspects. Chapter 1, a new chapter, presents an overview of the milk protein system, especially from an historical viewpoint. Chapters 2–5, 7–9, 15, and 16 are revisions of chapters in the second edition and cover analytical aspects, chemical and physiochemical properties, biosynthesis and genetic polymorphism of the principal milk proteins. Non-bovine caseins are reviewed in Chapter 6. Biological properties of milk proteins, which were covered in three chapters in the second edition, are now expanded to five chapters; a separate chapter, Chapter 10, is devoted to lactoferrin and Chapter 11, on indigenous enzymes in milk, has been restructured and expanded. Nutritional aspects, allergenicity of milk proteins, and bioactive peptides are discussed in Chapters 12, 13, and 14, respectively. Because of significant developments in the area in the last decade, Chapter 17 on genetic engineering of milk proteins has been included. Various aspects of the stability of milk proteins are covered in Chapter 18 (enzymatic coagulation), Chapter 19 (heat-induced coagulation), Chapter 20 (age gelation of sterilized milk), Chapter 21 (ethanol stability), and Chapter 22 (acid coagulation, a new chapter).

The book includes four chapters on the scientific aspects of protein-rich dairy products (milk powders, Chapter 23; ice cream, Chapter 24; cheese, Chapter 25; functional milk proteins, Chapter 26) and three chapters on technologically important properties of milk proteins (surface properties,

Chapter 27; thermal denaturation aggregation, Chapter 28; hydration and viscosity, Chapter 29).

Like its predecessors, this book is intended for academics, researchers at universities and industry, and senior students; each chapter is referenced extensively.

We wish to thank sincerely the 60 contributors to the 29 chapters of this volume, whose cooperation made our task as editors a pleasure. The generous assistance of Ms. Anne Cahalane is gratefully acknowledged.

Cork, Ireland

P.F. Fox
Paul L.H. Mcsweeney

Preface to the Second Edition

Considerable progress has been made on various aspects of milk proteins since *Developments in Dairy Chemistry 1—Proteins* was published in 1982. *Advanced Dairy Chemistry* can be regarded as the second edition of *Development in Dairy Chemistry* which has been updated and considerably expanded. Many of the original chapters have been revised and updated, e.g. 'Association of Caseins and Casein Micelle Structure', 'Biosynthesis of Milk Proteins', 'Enzymatic Coagulation of Milk', 'Heat Stability of Milk', 'Age Gelation of Sterilized Milks' and 'Nutritional Aspects of Milk Proteins'. Chapter 1 in *Developments*, i.e. 'Chemistry of Milk Proteins', has been subdivided and extended to 4 chapters: chemistry and physico-chemical properties of the caseins, β-lactoglobulin, α-lactalbumin and immunoglobulins. New chapters have been added, including 'Analytical Methods for Milk Proteins', 'Biologically Active Proteins and Peptides', 'Indigenous Enzymes in Milk', 'Genetic Polymorphism of Milk Proteins', 'Genetic Engineering of Milk Proteins', 'Ethanol Stability of Milk' and 'Significance of Proteins in Milk Powders'. A few subjects have been deleted or abbreviated; the three chapters on functional milk proteins in *Developments* have been abbreviated to one in view of the recently published 4th volume of *Developments in Dairy Chemistry—4— Functional Milk Proteins*.

Like its predecessor, the book is intended for lecturers, senior students and research personnel and each chapter is extensively referenced.

I would like to thank all the authors who contributed to the book and whose cooperation made my task a pleasure.

Cork, Ireland P.F. Fox

Preface to the First Edition

Because of its commercial and nutritional significance and the ease with which its principal constituents, proteins, lipids and lactose, can be purified free of each other, milk and dairy products have been the subject of chemical investigation for more than a century. Consequently, milk is the best-described in chemical terms, of the principal food groups. Scientific interest in milk is further stimulated by the great diversity of milks—there are about 4000 mammalian species, each of which secretes milk with specific characteristics. The relative ease with which the intact mammary gland can be isolated in an active state from the body makes milk a very attractive subject for biosynthetic studies. More than any other food commodity, milk is a very versatile raw material and a very wide range of food products are produced from the whole or fractionated system.

This text on Proteins is the first volume in an advanced series on selected topics in Dairy Chemistry. Each chapter is extensively referenced and, it is hoped, should prove a useful reference source for senior students, lecturers and research personnel. The selection of topics for 'Proteins' has been influenced by a wish to treat the subject in a comprehensive and balanced fashion. Thus, Chapters 1 and 2 are devoted to an in-depth review of the molecular and colloidal chemistry of the proteins of bovine milk. Although less exhaustively studied than those of bovine milk, considerable knowledge is available on the lactoproteins of a few other species and an inter-species comparison is made in Chapter 3. The biosynthesis of the principal lactoproteins is reviewed in Chapter 4. Chapters 5 to 8 are devoted to alterations in the colloidal state of milk proteins arising from chemical, physical or enzymatic modification during processing or storage, viz. enzymatic coagulation, heat-induced coagulation, age gelation of sterilized milks and chemical and enzymatic changes in cold-stored raw milk. Milk and dairy products provide 20–30% of protein in 'western' diets and are important world-wide in infant nutrition: lactoproteins in particular, are considered in Chapter 9. The increasing significance of 'fabricated' foods has created a demand for 'functional' proteins: Chapters 10 to 12 are devoted to the technology, functional properties and food applications of the caseinates and various whey protein products.

Because of space constraints, it was necessary to exclude coverage of the more traditional protein-rich dairy products: milk powders and cheese. It is hoped to devote sections of a future volume to these products.

I wish to thank sincerely the 13 other authors who have contributed to this text and whose cooperation made my task as editor a pleasure.

Cork, Ireland P.F. Fox

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Origin and Evolution of the Major Constituents of Milk

1

O.T. Oftedal

1.1 Introduction

Lactation is highly complex and apparently of ancient evolutionary origin (Oftedal, 2002a). The complicated signaling cross-talk among epithelial and underlying mesenchyme cells that is required for the differentiation, ductal branching, and proliferation of mammary tissue is only now being unraveled (Watson and Khaled, 2008), and the functional significance and patterns of expression of thousands of mammary genes are under investigation (Lemay *et al.*, 2009). Secreted milk is extremely varied in composition—from trace levels of fat in rhinos to more than 60% fat in some ice-breeding seals (Oftedal and Iverson, 1995)—and contains unique proteins (α_s-, β-, and κ_s-caseins, β-lactoglobulin, α-lactalbumin, whey acidic protein), membrane-enclosed lipid droplets, and sugars (lactose, milk oligosaccharides) that are not found elsewhere in nature. The fact that the major milk constituents are found across the spectrum of mammals, including the three main groups (monotremes, marsupials, and eutherians) that diverged in the Jurassic and/or Cretaceous, indicates that milk secretion was inherited from a pre-mammalian ancestor.

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The mammary gland and its secretion represent a major evolutionary novelty, without any known intermediates. In the mid-nineteenth century, the complexity and interdependence among mammary glands, milk, and dependent suckling young posed a challenge to Charles Darwin's theory of evolution by natural selection. Darwin rose to the challenge, devoting most of a chapter of the 1872 edition of *On the Origin of Species* to a discussion of the problems of evolutionary novelty, such as the origin of the eye and of the mammary gland. Over the years since, a variety of authors have speculated on the origin and evolution of lactation, trying to envision a process in which something so complex could have evolved step by step and be favored by natural selection (reviewed by Oftedal (2002a)). It is now clear not only that lactation is of ancient origin (Oftedal, 2002a; 2002b) but also that detailed study of milk constituents, and the genetic pathways by which they have evolved, can reveal much about the evolution of milk secretion (Rijnkels, 2002; Kawasaki and Weiss, 2003; Vorbach *et al.*, 2006; Sharp *et al.*, 2007; McClellan *et al.*, 2008; Lemay *et al.*, 2009; Lefevre *et al.*, 2010; Kawasaki *et al.*, 2011; Oftedal, 2012).

It is important to assess the evolution of milk within a broader evolutionary scope if we are to understand the functional significance of various steps along the way. Skin secretions may have been important to the tetrapods that were ancestral to the amniotes (ancestors of "reptiles," birds, and mammals), so a discussion of the

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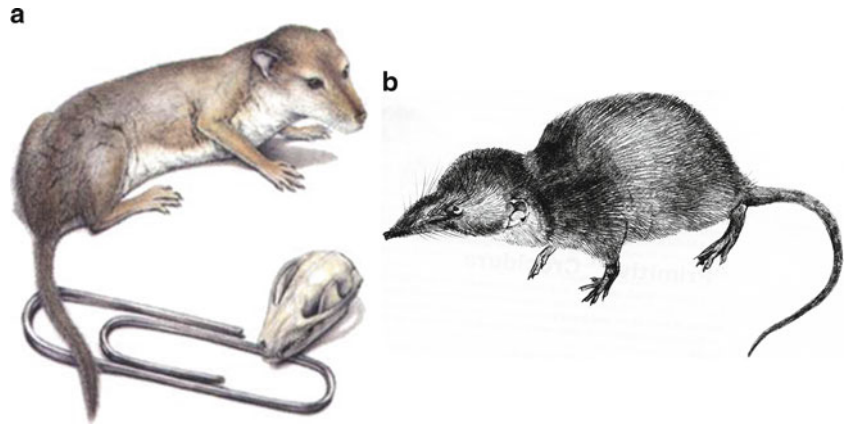

Fig. 1.1 Lactation at a very small size. (**a**) Lactation had to be far advanced for this small mammaliaform, *Hadrocodium wui*, from the early Jurassic (about 195 million years ago; Luo *et al*., 2001) to successfully reproduce. At a skull length of 1.2 cm and an estimated adult body mass of 2 g, the mother laid eggs that had to be tiny and the hatchlings consequently very immature. The hatchlings were probably reared on a milk rich in protein and high in energy density, as are contemporary shrews. (**b**) A living crocidurine shrew of about 10–12 g. At mid-lactation, *Crocidura russula* produces milk containing about 49% water, 30% fat, 9.4% protein, and 3% sugar (Mover *et al.*, 1985), but the young are live-born, not hatched from eggs (Credits: (**a**) Reproduced from the cover of Science Vol.292, no.5521, 25 May 2001. Reconstruction artwork: Mark A. Klingler, Carnegie Museum of Natural History. Reprinted with permission from AAAS; (**b**) Reproduced from Kingdon (1974), with copyright permission from Jonathan Kingdon)

evolution of lactation begins with them. I will briefly review the paleobiology of the ancestral forms that predate mammals, including the sequential radiations of amniotes, synapsids, therapsids, cynodonts, and mammaliaforms, as these taxa may have played a role in the evolution of lactation but are probably unfamiliar to most milk experts. Ironically, the evolution of milk is not so much a story about mammalian evolution, as a story that was largely complete before mammals appeared on the Earth (Fig. 1.1). The evolution of the synapsids and their descendents, of mammary glands, and of the eggs produced by synapsids has been treated more fully elsewhere (Oftedal, 2002a; b). A more recent perspective on these topics, and the evolution of specific milk constituents, is provided by Oftedal (2012). Substantial portions of the text of this chapter have been taken with permission from Oftedal (2012), sometimes with considerable elaboration.

In this chapter I follow the phylogenetic convention of applying taxon names to monophyletic groups, that is, to the ancestral form and all of its purported descendents. Thus if mammals evolved from mammaliaforms, they are a subset of mammaliaforms, and the synapsids that evolved from amniotes are a subset thereof. However ancestral forms can be distinguished by reference to time or geologic period. For ease of understanding, I use the term "reptiles" in quotation marks to refer to living lizards, snakes, turtles, and crocodilians, but excluding birds which are correctly nested within this group.

1.2 The Paleobiology of Lactation

The first vertebrates to set foot on land in the late Devonian (ca. 365 million years ago (mya)) were the tetrapods, a group ancestral to all subsequent terrestrial vertebrates (Fig. 1.2) (Carroll, 2009). The earliest forms still had skin that included bony scutes or dermal scales similar to that of the bony fish from which they evolved, at least on ventral surfaces; they may also have retained aquatic respiration despite skeletal modifications enabling locomotion on land. After a gap in the fossil record, a variety of forms appeared in the middle of the Carboniferous (ca. 345–315 mya), including smaller, more salamander-like forms (Carroll, 2009). The progressive reduction in dermal protection was presumably accompanied by the appearance of more rigid, mat-like webs of

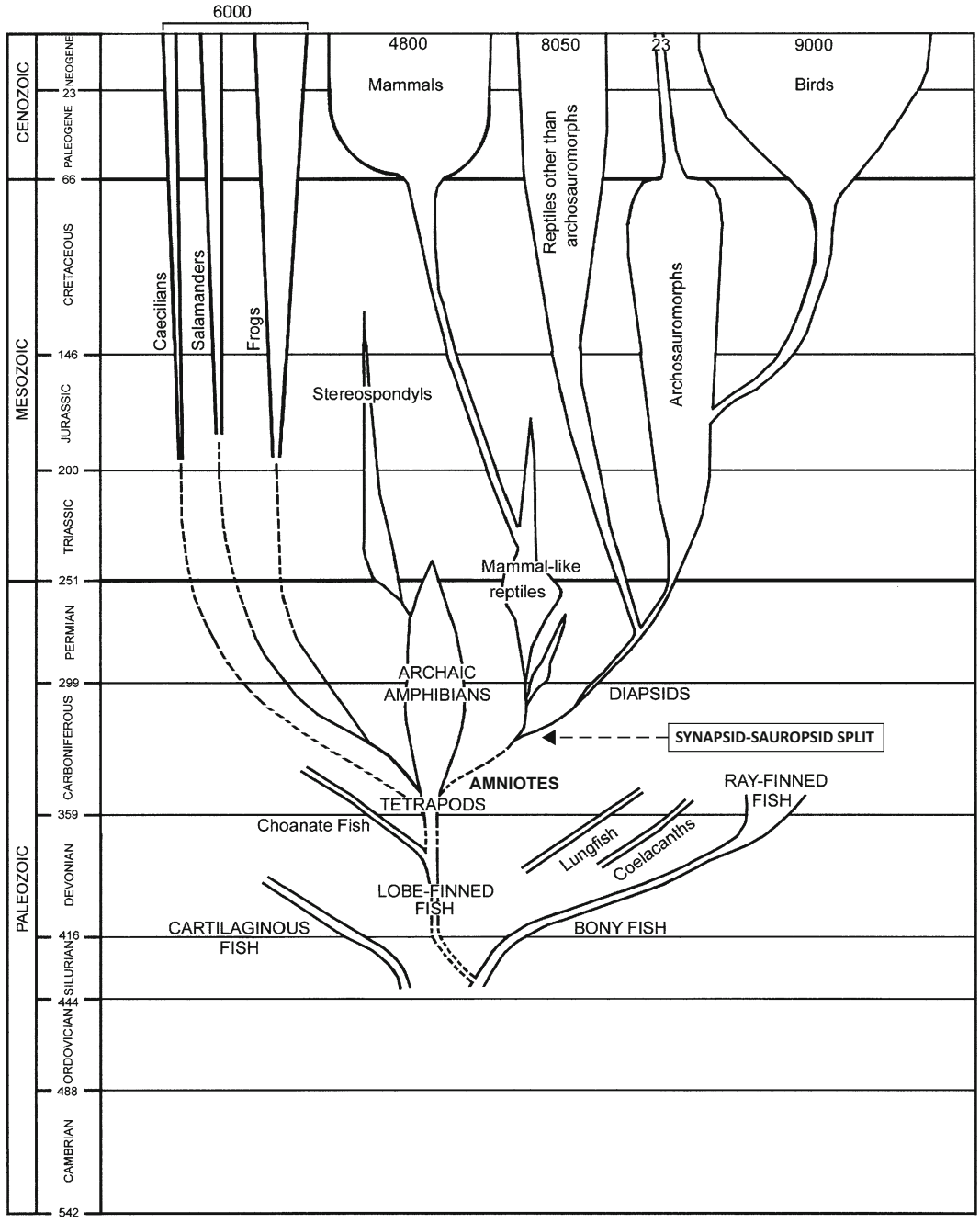

Fig. 1.2 The evolutionary history of terrestrial vertebrates, illustrating the divergence times of different lineages and their relative diversity (numbers of families, as indicated by width of lineages). Geologic periods are listed on *left* axis with transition dates in millions of years; approximate number of extant species listed at *top*. The split of the amniotes into synapsids (to the *left*, leading to mammals) and sauropsids (to the *right*, leading to "reptiles" and birds) is indicated by the *dashed arrowhead*. The so-called mammal-like reptiles, include sequential radiations of basal synapsids ("pelycosaurs"), therapsids, cynodonts, and mammaliaforms (not illustrated), ultimately leading to mammals (see Fig. 1.5). The archosauromorphs include dinosaurs and extant crocodilians, as well as birds (Credit: Reproduced from Carroll (2009), with copyright permission from Johns Hopkins University Press)

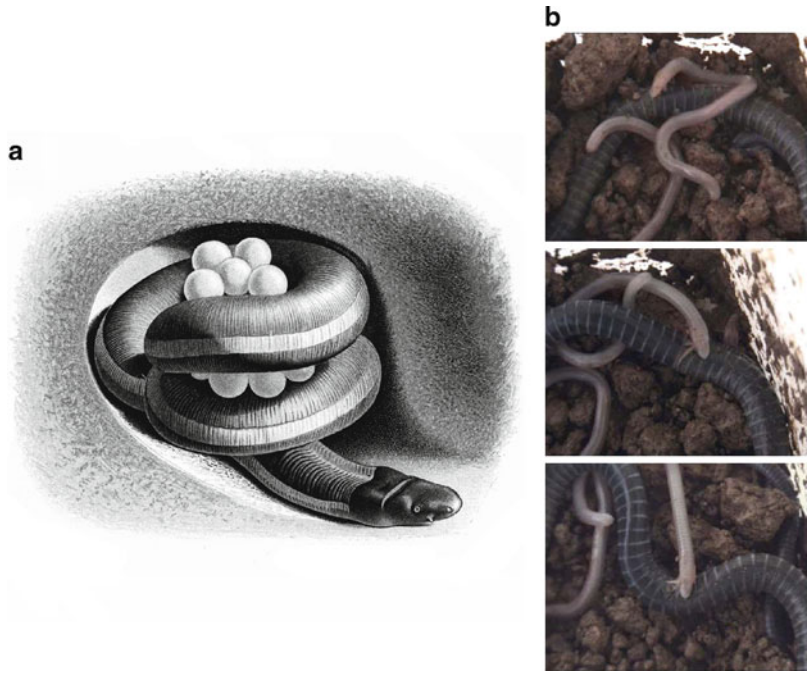

Fig. 1.3 Examples of maternal care in living amphibians. (**a**) Simple maternal egg brooding by an Asiatic caecilian *Ichthyophis glutinosus*, the presumed ancestral condition for terrestrial eggs. Nests of *Ichthyophis* sp. are usually in small subsurface chambers near water; females attend eggs for 3 months (Kupfer *et al.*, 2004). (**b**) Maternal provisioning of offspring in the caecilian *Boulengerula taitanus.* Maternal epidermal cells swell with vesicles containing lipids; hatchlings utilize specialized teeth to scrape skin, as demonstrated in these photographs by Alexander Kupfer taken in Kenya, January 2005. Sloughed skin and/or secretions appear to be consumed as the sole source of nutrients during early postnatal development in several caecilian taxa (Credits: (**a**) Reproduced from Sarasin and Sarasin (1887–1890); (**b**) Photographs used with copyright permission from A. Kupfer)

collagen in the skin for structural support, and the development of more elaborate skin glands that included toxic compounds to protect against infection and predation (Duellman and Trueb, 1994; Frolich, 1997; Oftedal, 2002a). The skin of tetrapods in the middle to late Carboniferous presumably resembled modern amphibians, such as frogs, salamanders, and caecilians, in having a relatively dense coverage of small multicellular secretory glands. Amphibian skin glands secrete primarily mucus (mucous glands) or bioactive constituents (granular glands) onto the skin surface (Clarke, 1997). In living amphibians, mucous glands play an important role in keeping the skin surface moist, facilitating exchange of respiratory gases (Lillywhite, 2006), while granular glands secrete a vast array of antimicrobial compounds: at least 500 antimicrobial peptides have been isolated from amphibian skin glands to date (Jenssen *et al.*, 2006). Some milk constituents, such as α-lactalbumin, β-lactoglobulin, whey acidic protein, and proteins in the mammary fat globule membrane, may originate from antimicrobial components that were expressed in Carboniferous tetrapod skin secretions as part of the innate immune system (see below).

Another feature that may have originated with tetrapods is parental care of terrestrial eggs (Fig. 1.3a), including the provision of secretions to keep them moist. The most primitive tetrapods are thought to have deposited eggs in fresh water where they were externally fertilized; these hatched into feeding larvae which, after a period of growth, underwent metamorphosis to produce semiterrestrial adults (Duellman and Trueb, 1994; Carroll, 2009). However, in all three living amphibian lineages (salamanders, frogs, and caecilians), a terrestrial system of egg development

has also evolved. In such amphibians, the eggs are macrolecithal (large-yolked), surrounded by multiple mucoid layers within capsules of oviducal origin, and develop directly into hatchlings without a larval stage. In such species, parental care is nearly universal (Fig. 1.3a) (Duellman and Trueb, 1994), and an important component of this care may be provision of supplemental moisture to the eggs via transcutaneous water movement on direct contact or via skin secretions (Taigen *et al.*, 1984). Among some caecilians, the maternal epidermis even swells with lipid-containing material after the young hatch from direct developing eggs, and the hatchlings use specialized "fetal teeth" to scrape this skin and/or secretions to obtain nutrients (Fig. 1.3b), comparable to ingestion of milk (Kupfer *et al.*, 2006). The Carboniferous tetrapods that subsequently evolved into amniotes (the lineage leading to mammals) presumably (1) were small (Carroll, 2009), (2) produced relatively large-yolked eggs with direct development (Packard and Seymour, 1997), (3) had glandular skin, and (4) engaged in parental care. If so, the trait of producing skin secretions to support egg development may have been inherited by the amniotes from earlier tetrapods.

The amniotes first appeared in the late Carboniferous (i.e., mid-Pennsylvanian, about 310 mya; Fig. 1.4). They are so termed because all of their surviving descendants ("reptiles," birds, and mammals) produce so-called amniotic eggs or did so prior to the evolution (in some lizards and most mammals) of uterine egg retention, placental structures, and birth of developed young. The amniotic egg was a major evolutionary novelty characterized by a fibrous eggshell (deposited in the oviduct), and a set of specialized extraembryonic membranes that served to partition and enhance various physiologic functions, such as respiration, waste storage, and osmotic interactions with the environment (Packard and Seymour, 1997; Stewart, 1997). The net effect was to permit eggs to become larger (i.e., contain more yolk to support development) and less dependent on moist conditions. However, the fibrous or parchment-like eggshell of the early amniotes lacked a superficial calcified layer such as later developed in "reptiles" and birds, and thus was still highly permeable to water, whether in liquid or gaseous form (Oftedal, 2002b). Such eggs are termed ectohydric, as they depend on uptake of environmental moisture for normal development. Thus it is possible, perhaps even probable, that the first amniote eggs were dependent on moisture provided by parents, just as in some terrestrial amphibians (Taigen *et al.*, 1984; Duellman and Trueb, 1994).

The earliest fossil amniotes include representatives of two lineages that subsequently evolved down different reproductive paths: the sauropsids (ancestors of living "reptiles" and birds) and the synapsids (ancestors of living mammals) (Fig. 1.5). The sauropsids continued to evolve features that allowed even greater independence of water: (1) the skin developed complicated epidermal scales including multiple layers of keratin and lipid, reducing skin moisture loss, (2) most secretory glands in the skin disappeared, except for specialized scent-marking glands (and in birds the preen gland), (3) the eggs developed calcified structures overlying the fibrous layer that greatly restricted moisture loss, and (4) in fully terrestrial species and birds, the primary waste product (uric acid) can be retained safely in the egg or excreted after hatching with minimal moisture loss (Packard and Packard, 1988; Oftedal, 2002a,b; Dhouailly, 2009). Living birds, crocodilians, many turtles, and some lizards produce endohydric eggs that contain all the water needed for development, primarily in an enlarged albumen layer. However, the synapsids continued to lay parchment-shelled eggs, retained a glandular skin, and if any evolved uric acid secretion as the primary nitrogenous waste product they did not leave surviving descendants (Oftedal, 2002a, b). A remarkable early Permian fossil of the integument of the synapsid *Estemmosuchus* (Therapsida: Dinocephalidae) includes a dense pattern of concave lens-like structures; Chudinov (1968) interpreted these as multicellular, flask-shaped alveolar glands, similar to the glands of amphibian skin; and argued that a glandular skin is a primitive synapsid feature still evident in mammals. Subsequently, different types of skin glands evolved. In mammals these now include eccrine, apocrine, and sebaceous glands, as well as complex scent glands, specialized glands providing secretions to eyes and ear canals, and mammary glands.

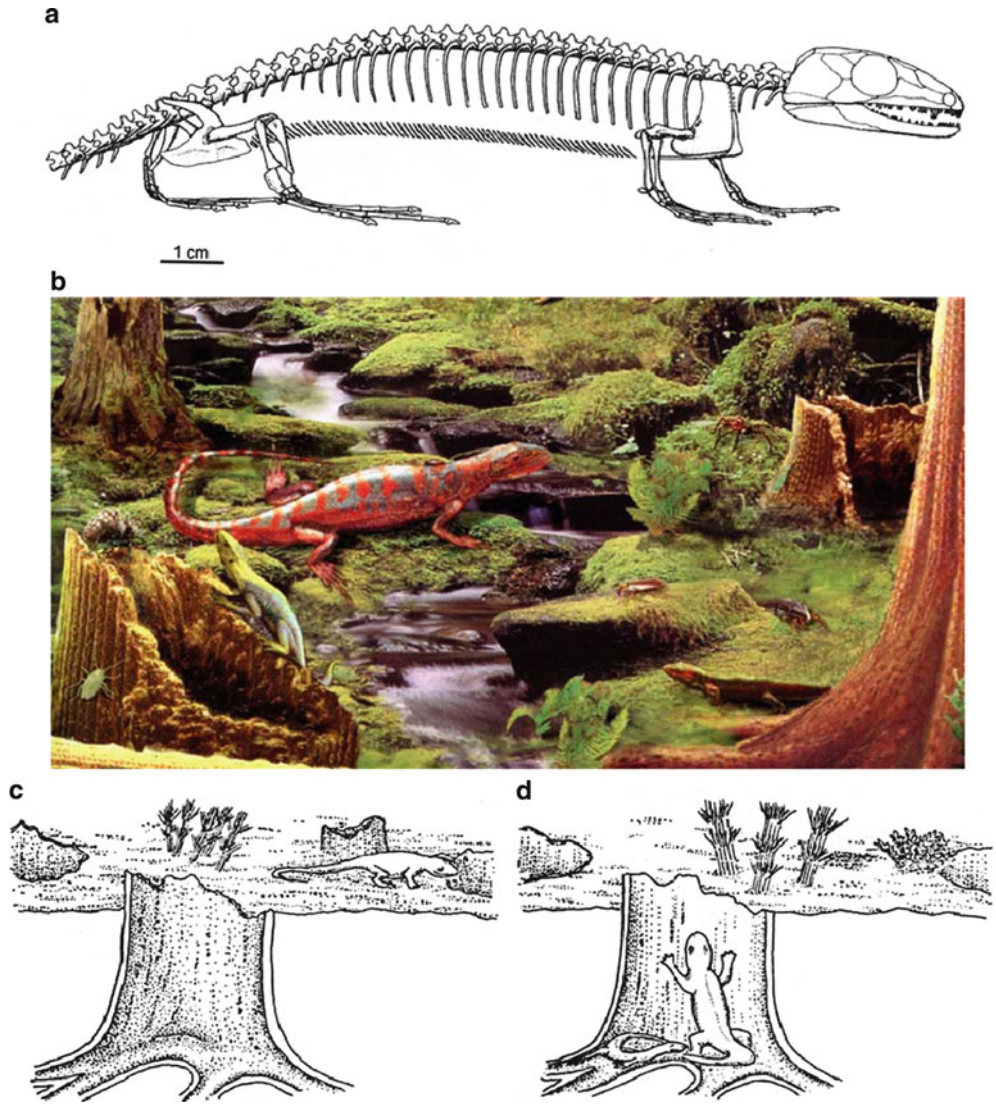

Fig. 1.4 The earliest amniotes from Carboniferous coal beds in Nova Scotia, Canada about 310 mya. (**a**) Reconstruction of skeleton of *Paleothyris* from Cape Breton Island. (**b**) Artist's conception of *Hylonomus* (*in center*) near stream surrounded by stumps of giant tree-like lycopods (clubmosses). (**c**, **d**) Schematic demonstrating presumed means of preservation of early amniotes: after a lycopod stump rotted out, the amniotes fell in, and became buried in sediment where they fossilized. As these egg-laying amniotes may also have exhibited parental care, it is conceivable that they were seeking nesting habitat (Credits: (**a**) Reproduced from Carroll (1969) with copyright permission from SEPM (Society for Sedimentary Geology); (**b**) Reproduced from Plate 7 (Artist: Tonino Terenzi) in Carroll (2009) with copyright permission from The Johns Hopkins University Press; (**c**) Reproduced from Carroll (1970) with copyright permission from Yale Scientific Magazine)

The eggs of early synapsids were likely large, well yolked, enclosed in a parchment-like eggshell, ectohydric, and subject to parental care; the hatchling produced was presumably small and capable of independent feeding (Oftedal, 2002b). Moisture was presumably transferred to eggs via transcutaneous osmotic transfer, from generalized skin secretions or from more specialized glandular regions. It is the latter that presumably evolved into mammary glands (Oftedal, 2002a).

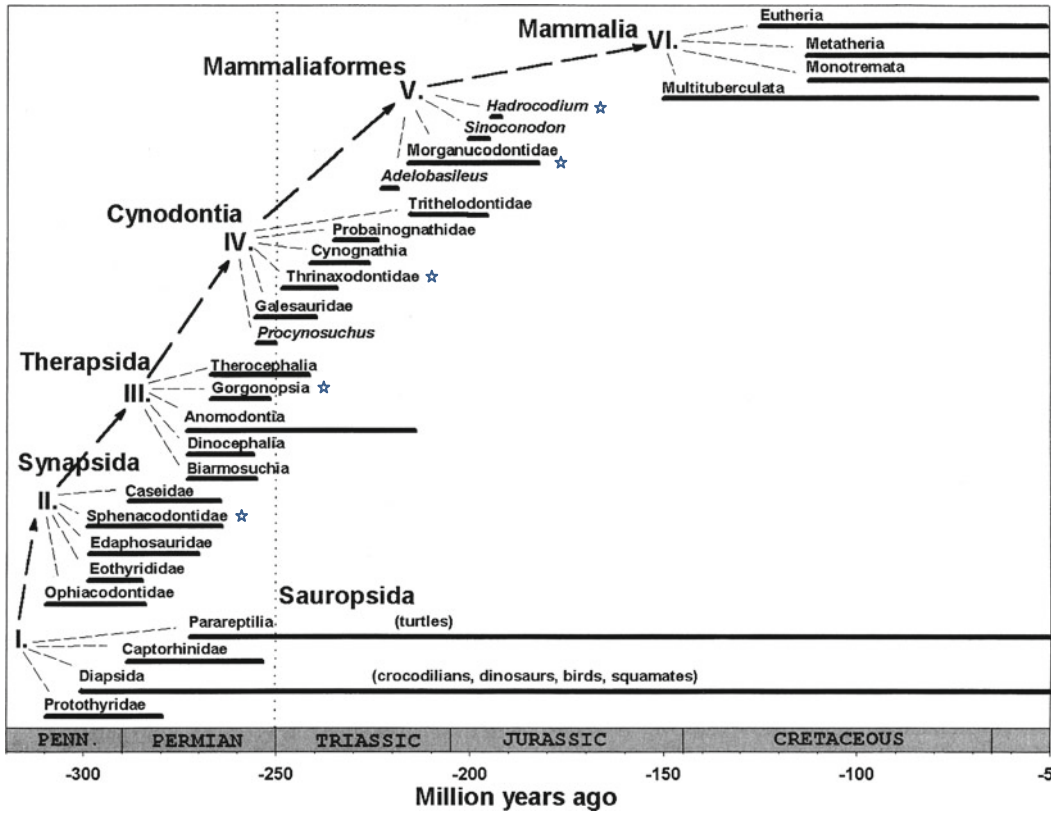

Fig. 1.5 Diagrammatic representation of sequential radiations beginning with Amniota (I) and concluding with Mammalia (VI). Note that each radiation derives from, and is a subset of, the preceding radiation. Some major and notable taxa within each radiation are illustrated, but many are omitted; those followed by *stars* are illustrated in Figs. 1.1 and 1.6. This representation does not include fossils described since 2002, including debates about which fossils represent the earliest eutherians and marsupial mammals (metatheria) (e.g., Wible *et al.*, 2007). Dates on *x*-axis are approximate; for more recent and precise transition dates between geologic ages, see Fig. 1.2 (Credit: Reproduced from Oftedal (2002a), with copyright permission from Springer Science and Business Media)

I have argued that lactation originated as a secretion that provided water, and other secretory constituents, to eggs (Oftedal, 2002b). Whether specialized "proto-lacteal" glands important to egg care first appeared among early tetrapods, or subsequently among synapsids, is not known. Molecular evidence suggesting that some milk constituents (including a proto-casein, α-lactalbumin, and β-lactoglobulin; see below) evolved before the origin of synapsids may indicate the former, but more research on gene expression in other glands and additional taxa is needed for clarification.

The synapsids subsequently underwent a series of extensive radiations followed by massive extinction events in which only a limited number of taxa survived into succeeding geologic periods as the basis for future radiations (Figs. 1.5 and 1.6) (Sidor and Hopson, 1998; Oftedal, 2002a; Kemp, 2005). Thus the basal synapsids (sometimes called "pelycosaurs" or "mammal-like reptiles"; Fig. 1.6a) radiated in the Pennsylvanian and early Permian, but most lineages went extinct by the mid-late Permian, when they were succeeded by a radiation termed therapsids (Fig. 1.6b). Most therapsid taxa disappeared during a massive extinction event at the end of the Permian, but the lineage of cynodonts survived to radiate in the Triassic (Fig. 1.6c). Most of these lineages in turn disappeared in the

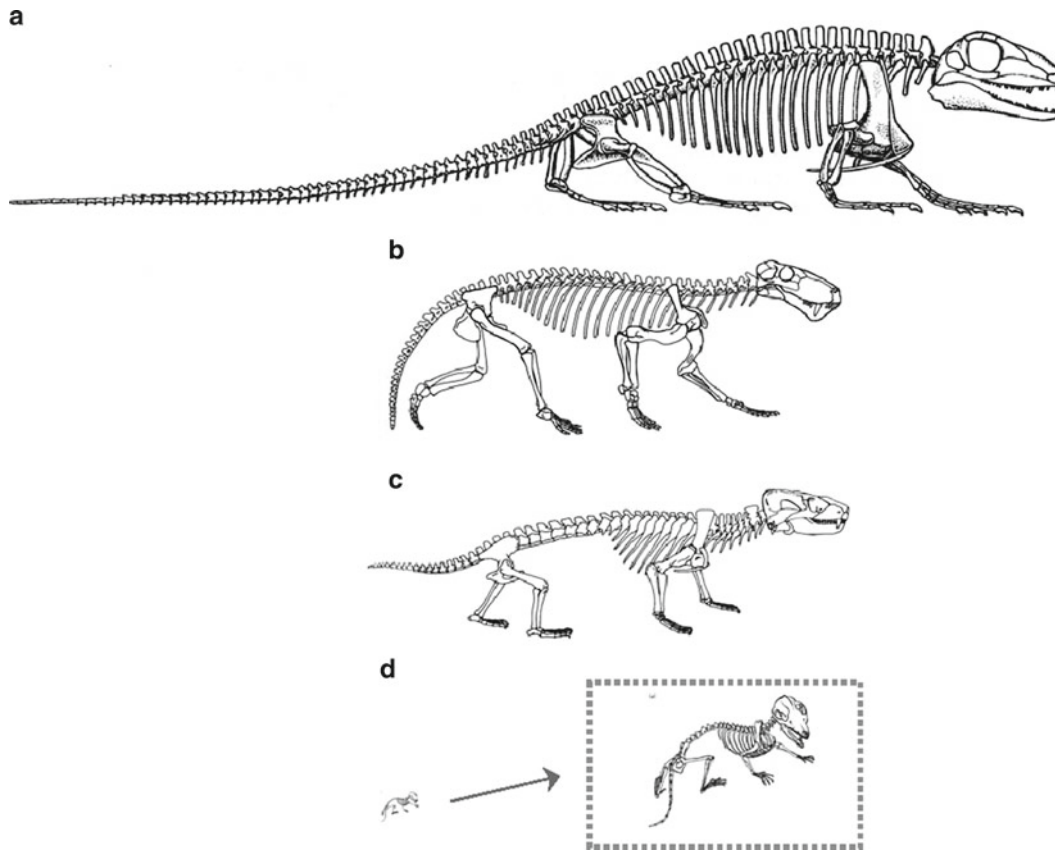

Fig. 1.6 Representatives of the sequential radiations of synapsids during the evolution of lactation. See Fig. 1.5 for time lines for these taxa. (**a**) *Haptodus*, an early synapsid carnivore in the Sphenacodontidae from the Pennsylvanian (= late Carboniferous) and early Permian, body length 2 m. (**b**) *Lycaenops*, a therapsid carnivore in the Gorgonopsidae, from the Permian, body length 1 m. (**c**) *Thrinaxodon*, a cynodont carnivore in the Thrinaxodontidae, from the early Triassic, body length 1 m. (**d**) *Morganucodon*, a mammaliaform carnivore in the Morganucodontidae, from the late Triassic, body length ca. 0.01 m (enlarged for visibility in dotted box) (Credits: (**a**) Reproduced from Currie (1977) with copyright permission from SEPM (Society for Sedimentary Geology); (**b**) Reproduced from Colbert (1948) with copyright permission from The American Museum of Natural History; (**c**) Reproduced from Jenkins (1984) with copyright permission from the University of Texas Department of Earth and Planetary Sciences; (**d**) Reproduced from Jenkins and Parrington (1976), with copyright permission from the Royal Society)

late Triassic, but a subset, the mammaliaforms, radiated in the late Triassic and Jurassic (Figs. 1.1 and 1.6d). It was from within the mammaliaforms that true mammals evolved, perhaps in the late Jurassic, about 160 mya (Fig. 1.5). These repeated radiations were undoubtedly important in the development of mammary glands and their secretions, but unfortunately only indirect evidence of such transitions are evident in the fossil record.

It is well established that the sequential radiations incorporated an increasing number of anatomic traits that now characterize mammals, that is, they became progressively more mammal-like (Sidor and Hopson, 1998; Kemp, 2005). Some of these traits involved changes in locomotion (from a sprawled lizard-like gait to an upright stance with improved running ability), in growth pattern (from a periodic pattern of bone mineralization to more continuous growth), in respiratory ability (reduction in ribs and development of diaphragmatic breathing), in heat exchange (using respiratory surfaces in the nasal cavity for

cooling and moisture retention), and in food-processing ability (rearrangement of skull and jaw bones for increased jaw musculature, diversification, and specialization of teeth and tooth cusps) (Oftedal, 2002a). Some of these changes can be seen in the sequence of carnivore skeletons illustrated in Fig. 1.6; however, there were also radiations of herbivores (not illustrated). The picture is one of increasing metabolic expenditure, increased growth rates, and increased activity—and a presumed upregulation of basal metabolism and increased refinement of body temperature regulation (i.e., development of endothermy or being "warm-blooded").

During the Triassic and early Jurassic, the cynodonts and mammaliaforms were not only developing elevated metabolic rates, they were also becoming progressively smaller in size (Kemp, 2005). This miniaturization of body size (Figs. 1.1 and 1.6d) would have required miniaturization of eggs as well. Even if eggs were kept in a pouch to prevent desiccation, once they hatched, the young would be too small to be effective homeotherms (Hopson, 1973). In birds, reduction of egg size in small species is accompanied by reduction of incubation time and hatching of altricial (incompletely developed) young (Starck and Ricklefs, 1998). Hopson (1973) argued that the diminutive mammaliaforms (some no more than a few g as adults; Fig. 1.1) must have been producing altricial young, as small eggs could not hold enough yolk to allow development of precocial (well developed) hatchlings. But altricial young would require feeding, indicating that lactation had already evolved.

Development of endothermy also required that eggs be incubated at or near body temperature. But unlike bird eggs, that were preadapted to endothermy by virtue of the extensive calcification of the shell that retarded moisture loss, synapsid eggs were highly susceptible to moisture loss when exposed to vapor pressure gradients, and thus could not have been incubated at an elevated temperature without an exogenous source of moisture (Oftedal, 2002b). This source was presumably a dilute milk, similar to the mammary secretions produced by monotremes (echidnas and the platypus) at the time of egg incubation; I even speculated that extant monotremes may still apply mammary secretions to incubating eggs and that this is one reason for the absence of nipples (which would interfere with such transfer) (Oftedal, 2002a, b). There is evidence in the regional specialization of the extraembryonic membranes in monotreme eggs that nutrient absorption could occur at the abembryonic end, that is, that respiratory and nutrient uptake functions are separated (Luckett, 1977; Oftedal, 2002b). One would not expect the entire egg or mammary patch to be wetted (contra Lefevre *et al.* (2010)), as this would drown the eggs. Water and nutrient transfer to incubated eggs could be demonstrated by isotope-labeling methods, but this has yet to be attempted. Unfortunately it is not possible to examine mammaliaform eggs, but I consider the coexistence of egg-laying and incipient endothermy as further evidence that lactation was well developed in the late Triassic.

This conclusion is bolstered by the fact that late cynodonts and the mammaliaforms developed a reduction in tooth replacement. Early synapsids, like most sauropsids, had teeth that were replaced continuously as an animal grew, allowing smaller teeth to be replaced by larger teeth as the jaw lengthened with age. Most mammals, by contrast, have only two sets of teeth—an initial set of deciduous "milk teeth" and the adult dentition. This developmental strategy, termed diphyodonty, is possible because tooth eruption is delayed while the jaw develops in utero and/or during the lactation period; there is no need for robust, adult-type teeth in dependent offspring that do not need to capture or consume an adult-type diet. Given that advanced placental structures did not occur until much later (after divergence of monotremes, marsupials, and eutherians), the fact that late cynodonts and early mammaliaforms already had evolved diphyodonty indicates that they were already reliant on milk for a substantial period of development (Oftedal, 2002a).

Thus a plausible scenario is that there were sequential stages in the evolution of lactation. Initially, a "proto-lacteal" secretion provided moisture, and antimicrobial constituents and

perhaps a few nutrients (such as calcium) to eggs. This may have occurred among tetrapods in the middle Carboniferous, or among early synapsids in the late Carboniferous and Permian. Both the secretions and the eggs presumably evolved to facilitate moisture and nutrient transfer and to counteract pathogenic attack. At some point, hatchlings, which were initially small but well developed, began to ingest the secretions, and the secretions evolved novel mechanisms for nutrient transport. As the reliance on mammary secretion increased, the investment of nutrients in egg yolk declined. Ultimately vitellogenins (lipoproteins important in egg yolk synthesis) themselves began to disappear in the Jurassic about 170 mya (Brawand *et al.*, 2008). It is likely that the presumed endothermy, minute adult size, and diphyodonty of mammaliaforms in the late Triassic were only possible because lactation was fully developed. This is supported by evidence that the major milk constituents are all pre-mammalian in origin; mammals first appeared and diversified in the late Jurassic about 160 mya (Luo and Wible, 2005).

Comparative analyses across monotreme, marsupial, and eutherian genomes indicate that milk and mammary genes are more highly conserved than other genes, presumably due to their functional importance to successful reproduction (Lemay *et al.*, 2009). Few new milk constituents have evolved since the origin of mammals, with the possible exception of some whey proteins in marsupials and perhaps the complex array of milk oligosaccharides observed in some mammals (but see section on Origin and Evolution of Milk Sugar Synthesis, below). On the other hand, some milk constituents appear to have lost ancestral functions as nutritive functions have become paramount. Among eutherian mammals the primary innovation has been the replacement of early lactation by placental nutrient transfer, allowing offspring to be born in a more or less advanced state of development, and perhaps allowing milk composition to become more specialized to particular life history and environmental features. The replacement of extended lactation by prenatal nutrient transfer is most extreme in species with large, highly developed neonates, such as some rodents (e.g., guinea pigs), small ruminants, seals, and cetaceans (Widdowson and McCance, 1955; Oftedal, 1985; Whitehead and Mann, 2000; Schulz and Bowen, 2005). At the extreme the hooded seal is so precocial at birth that lactation can be completed in just four days; as the milk is both very high in fat (>60%) and produced in voluminous amounts, the pup doubles its mass before weaning (Oftedal *et al.*, 1993). Hooded seal milk does not change in composition during lactation, in contrast to the remarkable changes in milk composition from hatching or birth to weaning in monotremes, marsupials, and eutherian mammals with altricial young (Oftedal and Iverson, 1995). Remarkable compositional change with lactation stage is undoubtedly the ancestral condition in mammals, as both mammaliaforms and the earliest mammals were apparently altricial at hatching, as are extant monotremes.

1.3 Origin and Evolution of Mammary Glands

With the proposed theory of evolution by natural selection, biologists questioned how such complex organs as mammary glands could have evolved from some simpler precursor. Darwin (1872) noted that mammary glands were homologous to cutaneous glands, and probably derived from them. Gegenbauer (1886) thought that the mammary glands of monotreme derived from "sweat glands," whereas those of marsupials and eutherians derived from sebaceous glands. Based on ontogenetic studies, Bresslau (1920) concluded that mammary glands derived from sweat glands associated with hair follicles. Blackburn (1991) expressed the view that multiple gland types may have contributed to mammary structure and function, considering the mammary gland a neomorphic hybrid. However, in a detailed review of the differences and similarities among gland types, I concluded that mammary glands are derived from an ancestral apocrine-like gland (Oftedal, 2002a).

Apocrine glands and mammary glands both secrete constituents by exocytosis of secretory vesicles and by a budding out and pinching off of cellular contents with loss of cytoplasm (Oftedal,

2002a). In apocrine glands, the latter process is considered an apocrine mode of secretion in contrast to merocrine secretion employing exocytosis of vesicles, or holocrine secretion in which cells swell with secretory product that is released via apoptotic disruption of cellular integrity. In mammary glands, budding out and pinching off occurs during secretion of the milk fat globule; cytoplasmic crescents may be present but are minimal (Mather and Keenan, 1998; Mather, 2011a). It is likely that milk fat globule secretion is a highly derived form of apocrine secretion in which upregulation of milk fat secretion has required incorporation of novel membrane constituents (see section on Origin and Evolution of the Milk Fat Globule, below). Unfortunately little is known about the details of secretion in generalized apocrine glands or about the genes expressed and proteins synthesized (Oftedal, 2002a), although they apparently do not include milk-specific proteins, such as β-casein (Gritli-Linde *et al.*, 2007). From an evolutionary perspective, it would be very interesting to compare the array of genes expressed by developing and secreting apocrine glands to those expressed during mammary gland development, milk secretion, and mammary involution. For example, nearly 200 milk protein genes and more than 6000 other genes have been identified as expressed in the mammary glands in virgin, pregnant, lactating, involuting, and mastitic cows (Lemay *et al.*, 2009), but how many of these genes are expressed in apocrine glands is unknown.

In most mammals, an apocrine gland on the general skin surface is typically associated with both a hair follicle and a sebaceous gland in a triad termed an apo-pilo-sebaceous unit (APSU) (Fig. 1.7a). The development of the APSU occurs in coordinated fashion, no doubt due to cross talk between the differentiating epithelial cells and underlying mesenchyme, as well as differences in signaling pathways and receptors of the hair follicle, apocrine gland, and sebaceous gland (Hatsell and Cowin, 2006; Andrechek *et al.*, 2008; Mayer *et al.*, 2008). The apocrine gland duct typically opens into the infundibulum of the hair follicle, such that secretion contacts the hair shaft (Fig. 1.7a). A parallel is found in monotremes in which mammary glands, hair follicles, and sebaceous glands form what can be termed a mammo-pilo-sebaceous unit (MPSU) (Oftedal, 2002a). Each galactophore (lactiferous duct) also opens up into the infundibulum of an enlarged, specialized mammary hair (Griffiths, 1978). The mammary glands in monotremes are organized into a small oval mammary patch or areola consisting of 100–200 MPSUs (Griffiths, 1978; Oftedal, 2002a); there is no nipple. In the area surrounding the mammary patch APSUs develop. Although the mature, lobular mammary gland in mid to late lactation is very much larger, more branched, and contains many more secretory epithelial cells than an apocrine gland, in earliest lactation, when monotreme eggs are incubated and hatched, the mammary gland is still relatively small and tubular (Griffiths, 1978), and thus has a superficial resemblance to an apocrine gland.

In marsupials, such as opossums and kangaroos, there is also a developmental association of mammary glands with hair follicles and sebaceous glands. According to early work by Bresslau (1912, 1920), an oval primary-primordium separates into nipple primordia which deepen into knobs and bud out into hair follicles (primary sprout), mammary glands (secondary sprout), and sebaceous glands (tertiary sprouts) (Fig. 1.7b). In the opossum, for example, eight hair follicle sprouts are associated with eight mammary sprouts and eight sebaceous sprouts, that is, the nipple primordium develops into eight MPSUs. The hair follicles penetrate the nipple epithelium during development but are subsequently shed, each leaving a duct (galactophore) by which the mammary gland communicates to the surface of the nipple (Fig. 1.7b). As opossums have a dozen or more nipples, about 100 MPSUs are involved. In the adult marsupial, the "mammary hairs" are no longer evident, but the galactophores bear testimony to their prior existence.

In eutherian mammals, apocrine glands retain an association with hair follicles (APSUs), but the association of mammary glands with hair follicles, the presumed ancestral condition, appears to have been lost. In 2002 I hypothesized that this must be due to inhibition of hair follicle development in the vicinity of mammary glands, and

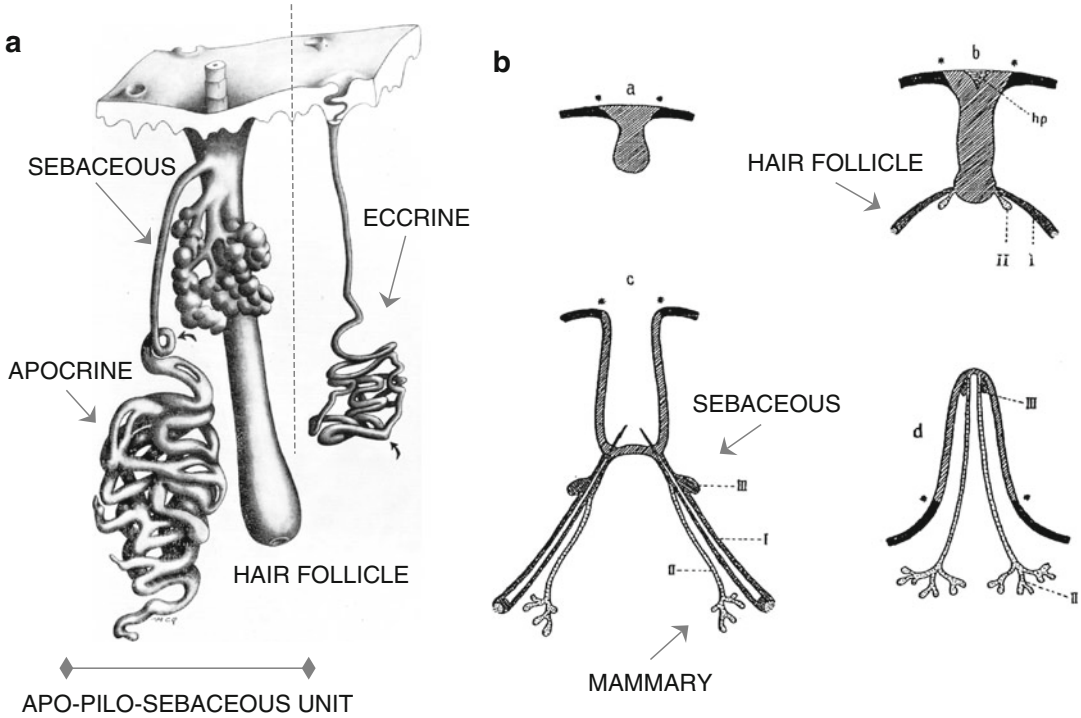

Fig. 1.7 The relationship among skin gland types in mammals. (**a**) On the general skin surface, the ducts of apocrine and sebaceous glands typically open into the infundibulum of a hair follicle, forming an apo-pilo-sebaceous unit (APSU), whereas eccrine sweat glands are separate. In the mammary patch of monotremes, a similar arrangement is seen, except mammary glands replace apocrine glands, forming a mammo-pilo-sebaceous unit with a distinctive mammary hair (see text). (**b**) Schematic representation of mammary gland ontogeny in kangaroos, and other marsupials that undergo nipple eversion, according to Bresslau (1912). In stage **a**, a nipple primordium forms, which in stage **b** elongates and a primary sprout (I) destined to become a hair follicle, and a secondary sprout (II) destined to become a mammary lobule, develop. In stage **c**, the nipple has hollowed out, producing a pouch-like structure, the hair follicle penetrates through it, the mammary sprout has become tubular, and a tertiary sprout (III) destined to become a sebaceous gland forms. Finally in stage **d**, the nipple has everted and the mammary hair has been shed, leaving a channel (galactophore), the sebaceous gland is associated with the everted nipple and the tubular mammary gland continues to proliferate (Credits: (**a**) Reproduced from Montagna (1962), with copyright permission from Elsevier B.V.; (**b**) Reproduced from Oftedal (2002a), with copyright permission from Springer Science and Business Media)

suggested that if the presumptive inhibiting compound(s) could be blocked at the earliest stages of mammary development, hair follicles might develop in association with mammary buds (Oftedal, 2002a). While the actual signaling pathways are undoubtedly complex, with both shared and differing sensitivities to signaling compounds among different epithelial cell types, it is now known that bone morphogenetic proteins (BMPs) inhibit hair follicle formation, and that when Mayer *et al.* (2008) reduced BMP signaling in the mouse by transgenic overexpression of a BMP antagonist, nipple epithelium was converted into pilosebaceous units. They hypothesized that the BMP pathway had been co-opted during evolution of the nipple to suppress hair follicle formation (Mayer *et al.*, 2008).

It has also been suggested that mammary secretion first developed as part of an inflammatory response by mucous secreting cells, on the basis that elements of the innate immune system (such as xanthine oxidoreductase) are incorporated into milk constituents and that certain signaling pathways of the innate immune system have a role in regulating mammary development (Vorbach *et al.*, 2006; McClellan *et al.*, 2008). Mammary

development certainly entails a type of branching morphogenesis driven by epithelial-mesenchymal interactions and involving coordinated development with stimulatory signaling in part from hepatocyte growth factor (HGF) and epidermal growth factor (EGF), balanced by inhibitory signaling from members of the transforming growth factor (TGF-β) family, but this type of morphogenesis is also found in tissues of more ancient evolutionary origin, such as the pancreas, lung, kidney prostrate, and salivary glands (Nelson and Bissell, 2006). The innate immune system itself is of even more ancient origin, with components shared among invertebrates and vertebrates (Beck and Habicht, 1996; Hoffmann *et al.*, 1999; Fujita, 2002). Thus the developmental pathways of the mammary gland probably derive from some preexisting tissue, but the incorporation of innate immune components into these pathways may be even more ancient. Extensive evidence suggests that this ancestral tissue was apocrine-like, associated with hair follicles and sebaceous glands, and subsequently co-opted for a new function, the secretion of a nutritive fluid for feeding of the young. However the apocrine-like glands themselves must derive, ultimately, from the simple glandular skin structures found in pre-amniote tetrapods (Quagliata *et al.*, 2006), whether from mucous glands (as suggested by Vorbach *et al.*, 2006), granular glands (which produce antimicrobial compounds, including innate immune constituents), or some currently unknown gland capable of apocrine lipid secretion. Some frogs secrete lipids as a means of reducing water loss across the skin (Lillywhite *et al.*, 1997; Lillywhite, 2006), but the glands involved have not been studied in detail.

Although the basic pattern of mammary development, and its regulation, may derive from a more ancient model, the extent of glandular proliferation and output, the remarkable repeated cycles of proliferation and secretion followed by cellular apoptosis and gland involution, and the types of secretory products formed represent evolutionary novelties. The evolution of lactation also involved the development of elaborate hormonal controls (Akers, 2002). It is intriguing that the neurohypophysial peptide hormone mesotocin, and its derived form (via a single amino acid substitution) oxytocin, came to play roles both in uterine contractions at parturition and the milk ejection response in response to suckling (Waverley *et al.*, 1988; Acher, 1996; Parry and Bathgate, 2000). Mesotocin is universally found among non-mammalian tetrapods (amphibians, "reptiles," birds) and may have a role in egg-laying (Takahashi and Kawashima, 2008); certainly, exogenous oxytocin is known to be effective in inducing oviposition in some taxa, such as turtles (Feldman, 2007), and related neuropeptides have been shown to stimulate oviposition in invertebrates (Kawada *et al.*, 2004). One can imagine that a hormone involved in regulating egg-laying might be co-opted during evolution into the role of inducing release of a secretion beneficial to those eggs.

1.4 Origin and Evolution of Caseins

The secretory products of the mammary gland represent the expression of a large number of genes that are upregulated during lactation, but many of these products remain unstudied (except as genes) or their functionalities are poorly understood (Smolenski *et al.*, 2007; Lemay *et al.*, 2009). On the other hand, some constituents are clearly very important to the nutrition of the offspring, such as the major milk proteins that provide essential and nonessential amino acids that serve as substrates for postnatal metabolism and are constituents of tissue proteins synthesized during growth.

Caseins are unique to milk, and as predominant proteins (along with a variety of whey proteins) they convey a large proportion of the amino acids that are required by the offspring. The caseins are phosphorylated during synthesis, and aggregate into large micelles containing calcium bound to phosphorus in calcium phosphate nanoclusters (Smyth *et al.*, 2004). Multiple caseins (characterized as α_s-, β-, and κ-caseins) participate in these micelles, but κ-casein plays a particularly important role in stabilizing the micelle in secreted milk. Caseins are thus a primary transport vehicle for calcium and phosphorus, essential minerals

needed by offspring for skeletal development, tissue growth and, especially in the case of phosphorus, for most aspects of cellular metabolism (e.g., as components of ATP and other phosphorylated high-energy compounds). Caseins also play a central role in digestive processes of suckling young. Once milk is ingested, κ-casein is vulnerable to digestive proteases, such as chymosin, and the release of a macropeptide from κ-casein destabilizes the entire micelle, leading to precipitation of the caseins as a gastric curd which entraps fat and is retained in the stomach. Caseins also precipitate as gastric contents become acidic (pH 4.6 or less, depending on species). Fat so entrapped is attacked by lipases (including milk, pregastric, and refluxed pancreatic lipases) while the caseins themselves are hydrolyzed by proteases. The evolution of a mechanism of converting a liquid (milk) to a solid (gastric curd) was no doubt important to the evolution of efficient digestive processes in suckling young.

All mammalian milks that have been studied contain the three primary types of caseins: α-, β-, and κ-caseins. Thus the caseins have a premammalian origin and had already diverged into the three primary types prior to the separation of monotremes, marsupials, and eutherians (Rijnkels, 2002, 2003; Lefevre *et al.*, 2009; Lefevre *et al.*, 2010). Subsequently there has been proliferation of additional α_s- and β-caseins via gene duplication and exon changes within the casein locus (Rijnkels, 2002; Lefevre *et al.*, 2009; Lefevre *et al.*, 2010). Although marsupials (represented by the opossum *Monodelphis domestica* genome) have only one α-casein gene (*CSN1*) and one β-casein gene (*CSN2*), monotremes (represented by the platypus genome) have one α_s-casein gene (*CSN1*) and two β-casein genes (*CSN2, CSN2b*), while eutherians (e.g., mouse, rat, cow, human) have two or three α_s-casein genes (*CSN1S1, CSN1S2A, CSN1S2B*) and one β-casein gene (*CSN2*) (Rijnkels, 2002; Lefevre *et al.*, 2009; 2010). The α_s- and β-caseins are considered calcium-sensitive, as they bind calcium and are precipitated by high calcium concentrations, and as such are functionally similar. These proteins have a loose, unfolded native configuration, and aside from the conserved signal peptide and phosphorylation sites required for calcium binding, they exhibit a high rate of amino acid substitution, presumably because interchange of amino acids at most sites disrupt neither structure nor function. By contrast, both the gene structure and amino acid composition of κ-casein are less variable, perhaps because of its unique role in stabilizing the micelle. All mammals studied to date have a single κ-casein gene comprised of five exons.

Despite the essential functional role of caseins in lactation, how they evolved from non-milk proteins has until recently been a mystery. The similarities in gene structure and function between and among α_s- and β-caseins led Ginger and Grigor (1999) and Rijnkels (2002) to propose that they had evolved from one ancestral casein gene via gene duplication, exon shuffling, and inversion. However, κ-casein seemed distinct. Similarities in amino acid sequence, location of cysteine residues, predicted secondary structure, and cleavage products formed during biological activity led to the suggestion that κ-casein and the γ chain of fibrinogen might have derived from a common ancestral gene (Jolles *et al.*, 1978; Ginger and Grigor, 1999), but if so, the transitional steps from one tissue and function to another were not clear. More recently, Kawasaki *et al.* (2011) failed to find much sequence identity or similarity in exon structure between platypus/opossum κ-casein and γ-fibrinogen and concluded that these two proteins are evolutionarily distinct.

It is now apparent that the caseins are members of a much larger family of proteins of unfolded nature that are secreted from cells, usually in association with tissue mineralization or regulation of calcium at target tissues. These proteins, termed secretory calcium-binding phosphoproteins (SCPP), are secreted by secretory epithelial cells or cells derived from underlying ectomesenchymal cells, and have an ancient history in the evolution of mineralized vertebrate tissues (Kawasaki and Weiss, 2003; Kawasaki, 2009). The SCPPs include extracellular matrix proteins secreted by ameloblasts, odontoblasts, and osteoblasts—that function in the development of mineralized structures in enamel, dentin,

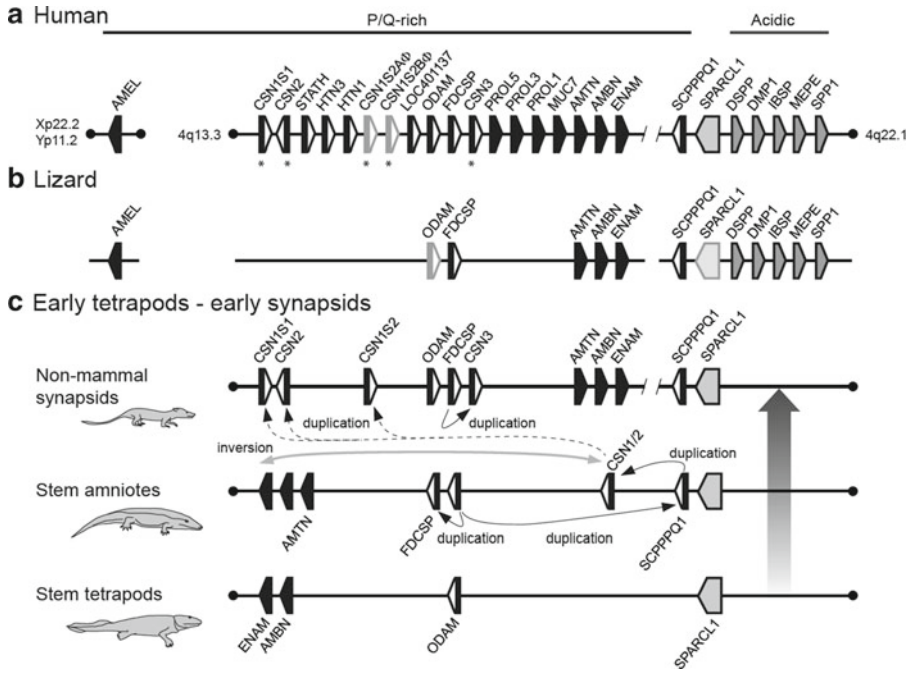

Fig. 1.8 Comparison of chromosomal locations and hypothetical duplications and inversions in the evolution of SCPP (including casein) genes from stem tetrapods to mammals, per Kawasaki *et al.* (2011). Each pentagon illustrates a gene and its transcriptional direction. See text for names of some P/Q-rich SCPPs; others are discussed in Kawasaki *et al.* (2011). P/Q-rich SCPPs with an entirely untranslated last exon are shown in black with a white tail and are presumably related via gene duplication, as in the hypothesized duplication of *SCPPPQ1* to produce the ancestral *CSN1/2* gene for α-/β-casein synthesis before or around the time of origin of amniotes. Note that the *CSN3* gene for κ-casein synthesis is hypothesized to arise from the *FDCSP* gene (Credit: Reproduced from Kawasaki *et al.* (2011), with copyright permission from Oxford University Press)

and bone, respectively—as well as salivary proteins that bind and transport calcium. As unfolded proteins, all SCPPs are low in cysteine (and hence cystine disulfide bridges) and a subclass of the proteins (P/Q-rich SCPPs), including the caseins, are particularly rich in proline and glutamine (Kawasaki and Weiss, 2003; Kawasaki *et al.*, 2011). It may also have evolutionary significance that a set of P/Q-rich milk and salivary proteins are translated from SCPP genes in which the last exon is entirely untranslated, and most of these are located within a gene cluster encompassed by the *CSN1S1* gene at the 5′ end and *CSN3* gene at the 3′ end (Fig. 1.8).

None of the milk casein genes has been found in sauropsids, but two members of this gene cluster, *ODAM* (which codes for odontogenic, ameloblast-associated protein) and *FDCSP* (which codes for follicular dendritic cell-secreted peptide) have been found in the genomes of a frog (*Xenopus*) and a lizard (*Anolis*), respectively (Kawasaki *et al.*, 2011). In addition, another structurally similar gene *SCPPPQ1* (secretory calcium-binding phosphoprotein proline-glutamine-rich-1 gene) that is currently located outside this cluster (but that, according to Kawasaki *et al.* (2011), was adjacent to *ODAM* in the stem amniote) has been found in the lizard genome (Fig. 1.8). Based on the relative locations and structures of exons of these P/Q-rich SCPPs, as well as their phylogenetic distribution, Kawasaki *et al.* (2011) propose (Fig. 1.8) that the α_s- and β-caseins derive via gene duplication and exon changes from an ancestral gene (*CSN1/2*) that derives from another SCPP gene, either *ODAM* or *SCPPPQ1* (which itself derived from *ODAM*), while κ-casein derives from the SCPP gene *FDCSP* (which also derived from *ODAM*). If this

scenario is correct, the *ODAM* gene is ultimately the grandmother of all caseins, and thus played a central role in the evolution of synapsid reproduction.

Identifying the biologically significant events in such a transformation is speculative and subject to revision as more information is gained about the phylogenetic distribution, tissue-specific expression, and functional roles of the secreted products of these SCPP genes. Initially Kawasaki and Weiss (2006) proposed that a primordial casein expressed by an ancestral casein gene might have had an antimicrobial role in skin secretions, perhaps protecting parchment-shelled eggs from microorganisms. They attributed an antimicrobial function to extant caseins, but such antimicrobial activity is by peptides produced from caseins during their digestion, not by intact proteins (Clare and Swaisgood, 2000). It is possible that evolution favored the development of antimicrobial attributes in products of casein proteolysis as a defense against microbial attack, whether in the secretory gland, on the skin surface, on an egg surface, or after ingestion by hatchlings, but this does not explain the original functional role of intact caseins.

More recently Kawasaki *et al.* (2011) propose that it is the calcium binding of an ancestral SCPP that was critical. Many P/Q-rich SCPPs, including the *ODAM* and *SCPPPQ1* proteins, are expressed in mammalian ameloblasts and are involved in mineralization of tooth enamel; FDCSP is found in soft connective tissue (periodontal ligament), where it is thought to prevent spontaneous precipitation of calcium phosphate, and is also expressed in the mammary gland (Kawasaki, 2009; Kawasaki *et al.*, 2011). Kawasaki *et al.* (2011) suggest that the initial function of an ancestral SCPP (probably a κ-casein precursor) in a proto-lacteal secretion may have been to regulate calcium delivery to the surface of an egg and to prevent precipitation of calcium phosphate on the eggshell. Kawasaki *et al.* (2011) hypothesize that this may have occurred prior to the divergence of sauropsids and synapsids, although an ancestral *CSN1/2* has yet to be found in a tetrapod or sauropsid genome (Fig. 1.8). In this context it might be informative to examine SCPP genes and their products in a broader range of living amphibians, including live-bearing caecilians in which the young obtain nutrients from maternal skin secretions and/or by ingestion of maternal superficial skin layers (Fig. 1.3) (Kupfer *et al.*, 2006). The substantial growth of the offspring from birth to independence indicates that they obtain calcium from their mothers, and the fact that the young utilize specialized fetal teeth to scrape maternal skin predicts that SCPP proteins may already be of developmental importance.

A role of skin-secreted calcium in delivering calcium to eggs is consistent with the view that early amniotes (predecessors of sauropsids and synapsids) produced eggs with a fibrous calcium-free eggshell (Packard and Seymour, 1997), that such eggs can utilize environmental calcium (and other minerals, such as sodium) (Thompson *et al.*, 2000), and that limited calcium supply in yolk might make this beneficial (Oftedal, 2002a, b). One of the features of the small tetrapods that evolved into amniotes was earlier and more extensive skeletal calcification, indicating that calcium supply may have become increasingly important (Carroll, 2009). There is a substantial literature on the role of different sources of calcium (eggshell, yolk, and environment) in different sauropsids (Packard and Clark, 1996; Stewart and Ecay, 2010). In species in which parchment-shelled eggs are retained in the uterus during part or all of development substantial calcium uptake may occur across the eggshell (Thompson *et al.*, 2000; Ramirez-Pinilla, 2006; Stewart and Ecay, 2010). There is also evidence that extraembryonic membranes in parchment-shelled eggs utilize calbindin-D_{28K} to assist in epithelial calcium transport, and that calbindin-D_{28K} concentrations are highest in the chorioallantoic membrane at the abembryonal end of the egg, suggesting regional specialization for calcium uptake (Ecay *et al.*, 2004). The bilaminar omphalopleure membrane at the abembryonal end of monotreme eggs and in "retained" marsupial eggs may also have a special role in nutrient uptake (Luckett, 1977; Tyndale-Biscoe and Renfree, 1987; Oftedal, 2002b). Thus a postulated role of casein

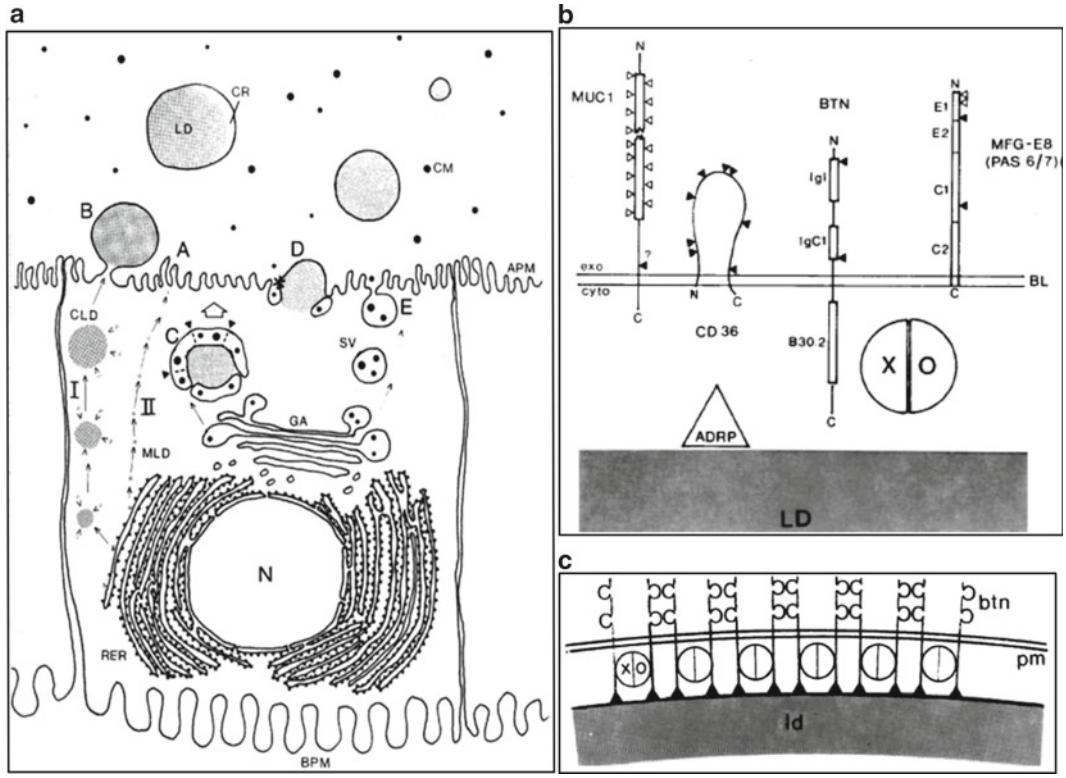

Fig. 1.9 (**a**) The milk fat globule and its membrane. (**A**) Schematic indicating bulging out (**D**) and pinching off (**B**) of lipid droplets (LD) as membrane-bounded milk fat globules containing cytoplasmic crescents (CR) at the same time as casein micelles (CM)and other constituents are being released from secretory vesicles (SV) into the alveolar lumen by exocytosis (**E**). Lipid droplets are synthesized as microlipid (MLD) or cytoplasmic lipid droplets (CLD) which increase in size during migration to the apical plasma membrane (APM). Other cell organelles include rough endoplasmic reticulum (RER), Golgi apparatus (GA), and basement plasma membrane (BPM). For more detail and explanation see Mather and Keenan (1998) and Mather (2011b). (**b**) Schematic indicating a model of the protein constituents of the milk fat globule membrane (MFGM) and their relation to the phospholipid bilayer (BL) and the enclosed lipid droplet (LD). Proteins embedded in the BL include mucin1 (MUC1), which projects into the exoplasmic fluid (exo) and is heavily glycosylated (*open triangles* indicate *O*-linked glycans), CD-36 which is also largely exoplasmic and glycosylated (*closed triangles* indicate N-linked glycans), butyrophilin1A1 (BTN) which includes exoplasmic immunoglobulin-like domains as well as a cytoplasmic B30.2 domain, and the exoplasmic PAS 6/7 glycoprotein. Xanthine oxidoreductase (XO) is cytoplasmic, but associates with the B.30.2 domain of BTN. Adipophilin (ADRP) is believed to associate with the surface of the lipid droplet. (**c**) Proposed Mather and Keenan model showing binding or close association of BTN, XOR (*circles*) and adipophilin (*solid triangles*) thereby minimizing the cytoplasmic space between the plasma membrane (PM) and lipid droplet, which may have been essential to evolutionary upregulation of milk lipid secretion without excessive cytoplasmic loss (see text) (Credits: All illustrations reproduced from Mather and Keenan (1998), with copyright permission from Springer Science and Business Media)

precursors in calcium delivery to late tetrapod or early amniote eggs appears feasible.

Calcium transport and surface regulation by an ancestral casein would just be the first step towards the much greater nutrient fluxes that must have evolved to feed hatchlings. By gene duplication and exon changes, the types of caseins and the numbers of genes involved in producing each type increased (Rijnkels, 2002; Lefevre *et al.*, 2009; Lefevre *et al.*, 2010; Kawasaki *et al.*, 2011). The different caseins became associated, perhaps first as an amorphous aggregate with sequestered amorphous calcium phosphate (Holt and Carver, 2012), but subsequently by the formation of complex micelles stabilized by calcium and phosphate bonds (Smyth *et al.*, 2004). This transformation of

ancestral SCPP protein(s) into a complex of micelle-forming proteins was essential in converting milk from an egg supplement to a major source of nutrients for suckling young. Given the small size of mammaliaforms in the late Triassic and Jurassic (Figs. 1.1 and 1.6d), and hence the small size of their eggs (Hopson, 1973; Oftedal, 2002a), the novel nutritive function of these SCPPs must have developed before this time, for example, during the Permian and Triassic.

The predominance of caseins as nutrient transporters to the young is also evident in the progressive loss of the ability to express vitellogenins. Vitellogenins are large multi-domain lipoproteins synthesized in sauropsid liver and transported to the ovaries where they are endocytosed and cleaved to produce the major egg yolk proteins, such as lipovitellins, phosvitin (a phosphoprotein), and β-component (Finn, 2007). The tetrapod ancestor of amniotes apparently coded for vitellogenins via two genes, *VIT1* and VIT_{anc}, but VIT_{anc} duplicated so that early amniotes had three *VIT* genes: *VIT1*, *VIT2,* and *VIT3* (Brawand *et al.*, 2008). During synapsid evolution, these genes became sequentially inactivated by insertion/deletion (indel) events, as well as by base substitutions that generated stop codons. Brawand *et al.* (2008) were able to recover an intact VIT gene (identity uncertain) in monotremes, which is consistent with continued egg-laying. Based on analysis of indel and stop codon rates, Brawand *et al.* (2008) estimated that in marsupials and eutherians *VIT3* had been inactivated about 170 mya (95%CI = 110–240 mya), *VIT1* about 140 mya (95% CI = 90–200 mya), and *VIT 2* about 70–90 mya (marsupial lineage only; unfortunately, *VIT2* pseudogenes have yet to be recovered from eutherian genomes). Vitellogenin genes could only be inactivated once a well-developed nutrient transport function by the caseins had made egg yolk proteins dispensible (Brawand *et al.*, 2008). The estimated inactivation of *VIT3* in the Jurassic indicates a loss or redundancy of the nutritional transport role of this vitellogenin in mammaliaforms (Fig. 1.5), which is consistent with their small size, small eggs, and purported dependence on milk. As caseins and other milk constituents continued to be the primary nutrient sources for the young, *VIT 1* became inactive about the end of the Jurassic, while *VIT2* became inactive (in marsupials) in the late Cretaceous. The timing of these events suggest (but do not prove) that marsupials continued to produce yolked eggs well after diverging from eutherians. This is not surprising because the developing young of some marsupials, including brush-tailed possums and koalas, still develop vestigial egg teeth (Hill, 1949).

1.5 Origin and Evolution of the Milk Fat Globule

An essential feature of milk is that it supplies energy-containing substrates in sufficient amounts to developing offspring that they are able to sustain metabolic requirements of the body and its component organs (such as the brain) while allowing amino acids, phospholipids, neutral lipids, and other energy-containing constituents to be invested in the development and proliferation of new tissues. In eggs the principal energy constituent is yolk, and in particular the lipids contained in the yolk. Lipids are energy dense and, because of their hydrophobic nature, can be packaged into compact structures that contain little water. However, creating a stable emulsion of fat in water that could be transferred to the young in fluid form required the evolution of a specific method of milk lipid secretion. The process of milk lipid secretion appears to be unique; it is not found in other organs that have been examined (McManaman *et al.*, 2006), and appears to be a key evolutionary novelty of the mammary gland (or its antecedent secretory glands).

Mammals vary tremendously in the fat content of their milk (from less than 1% in rhinos and some lemurs to 60% in some seals (Oftedal and Iverson, 1995), but in all species studied, milk lipids are packaged into specialized structures known as milk fat globules (Fig. 1.9a). Milk fat globules (MFG) are lipid spheres bounded sequentially by a phospholipid monolayer, an inner protein coat, a bilayered phospholipid membrane, and a glycosylated surface (Mather and Keenan, 1998). Transmembrane proteins such as mucins, butyrophilin, and CD36

(Fig. 1.9b) may interact both with proteins of the inner coat, such as xanthine oxidoreductase, fatty acid-binding protein and adipophilin, and with molecules at the milk-facing surface, via the domains or amino acid sequences that project inward and outward beyond the bilayered membrane, respectively (Mather, 2011a). The glycosylated surface is primarily due to oligosaccharide chains attached to outwardly projecting domains of the mucins and other transmembrane proteins. The collective term for the multilayered structure or envelope that encloses the lipid sphere is the milk fat globule membrane (MFGM), which is a structure known only from milk.

The secretion of lipid in such unique, highly organized membrane-bound packets that remain suspended in fluid is certainly a very different mechanism than the release of the contents of secretory vesicles by exocytosis as occurs in many secretory cells (including mammary secretory cells, or lactocytes; Fig. 1.9a), or the swelling of secretory cells with product and their release via lysis into the gland lumen, as during lipid secretion by sebaceous glands (Oftedal, 2002a). Although current understanding of the secretion, structure, and function of MFG constituents comes primarily from studies of human, ruminant, and rodent milks (McManaman, 2009; Mather, 2011a), these observations probably apply generally to milk lipid secretion, given the apparent similarities in the secretory mechanism and ultrastructure of MFGs across a wide range of species, including monotremes, marsupials, and the highest milk fat producers, the phocid seals (Griffiths *et al.*, 1973; Griffiths, 1978; Tedman, 1983).

The synthesis and accumulation of lipids within lactocytes has been reviewed by McManaman (2009). Triacylglycerols destined for secretion in the milk fat globule are initially synthesized by lipogenic enzymes associated with the endoplasmic reticulum, and appear as cytoplasmic lipid droplets (and small microlipid droplets) in the cytoplasm (Fig. 1.9a). These droplets are coated with a monolayer of phospholipids to which both structural proteins and enzymes are bound. As they migrate towards the apical surface of the cell, they increase in size, either via fusion of small droplets to produce larger droplets or simply by swelling as additional lipids are acquired from cytosolic transport proteins and/or via lipogenic activity by enzymes associated with the droplets (Mather and Keenan, 1998; McManaman, 2009; Mather, 2011b). At the cell surface, larger cytoplasmic droplets bulge out through the apical membrane and are then "pinched off" (Fig. 1.9a). In this process they become wrapped in MFGM that is believed to derive primarily from the apical plasma membrane of the secretory cell, but is augmented by proteins that form an interface between the surface of the lipid spheres and the inner surface of the bilayered membrane. The bulging out and pinching off of the mammary fat globule can also entrap some cytoplasm, which appears as "cytoplasmic crescents" in two-dimensional images of milk fat globules.

The secretion of the milk fat globule and its associated MFGM envelope is a highly regulated process that requires the presence and incorporation of specific components. What is of particular interest is that the MFGM contains proteins (Fig. 1.9b) that appear essential to the synthesis and secretion of milk fat globules. In particular, two proteins, butyrophilin and xanthine oxidoreductase (XOR), play an obligatory structural role in MFGM synthesis, and if they are reduced or eliminated from mouse mammary cells via knockout of the genes that code them, mice fail to produce normal milk; the triacylglycerols within the secretory cells fail to be secreted into milk fat droplets, but rather accumulate in the cytoplasm or leak into the alveolar lumen as unstructured, amorphic lipid masses (Vorbach *et al.*, 2002; Ogg *et al.*, 2004). While the details of protein-protein interactions during formation of the MFGM are not fully understood (Mather, 2011a), these two proteins have apparently been co-opted from other cellular functions during the evolution of the mammary gland and thus may be key to understanding the evolution of mammary fat secretion.

The butyrophilin in milk is now correctly specified as butyrophilin1A1, since it is the gene product of only one of the genes (BTN1A) that code for the family of proteins known as butyrophilins (Rhodes *et al.*, 2001). Three distinct

butyrophilin coding genes (*BTN1*, *BTN2,* and *BTN3*) have been located in the extended major histocompatibility complex region of the human genome, but due to double duplication of a chromosomal block that contained *BTN2* and *BTN3*, 3 gene copies exist for both *BTN2* and *BTN3* (Rhodes *et al.*, 2001). The butyrophilins are part of the immunoglobulin superfamily and are similar in structure to receptor proteins (B7.1 and B7.2) on antigen-presenting cells involved in the stimulation of T cell leukocytes. They contain two folded immunoglobulin domains, a transmembrane domain and a C-terminal end that may include a large B30.2 domain that is structured as a β-sandwich with presumptive protein binding sites. In butyrophilin1A1 in the MFGM, the Ig domains project outward from the bilaminar membrane into the alveolus (or milk), the transmembrane region straddles the bilaminar membrane, and the B30.2 domain projects inward into the underlying protein coat (Fig. 1.9b) where it binds XOR with high affinity, which may be important to its role in MFGM synthesis (Jeong *et al.*, 2009; Mather, 2011a). In addition to its role in the MFGM, butyrophilin1A1 has been found to be expressed within the thymus; low levels of transcription may be observed in other tissues but the degree of protein expression is not clear. Other butyrophilins are more widely expressed among tissues (Smith *et al.*, 2010a). Butyrophilins and related proteins of the immunoglobulin superfamily appear to play a role in regulation of proliferation, cytokine secretion, and activity of T cells, and butyrophilin1A1 retains this function, at least in vitro (Smith *et al.*, 2010a). It appears that the ancestral butyrophilin protein was a transmembrane protein in secretory cells that had functions in local immune response, and subsequently evolved a role in synthesis and/or stabilization of the MFGM.

It is interesting that butyrophilin1A1 is the only butyrophilin that appears to bind XOR via its B30.2 domain, and it this binding that is believed to be critical to milk fat globule secretion from lactocytes (Jeong *et al.*, 2009). While the biochemical sequelae and ultrastructural consequences of butyrophilin1A1-XOR binding are not certain, it is hypothesized that XOR may serve to link to other proteins of the protein coat and that these interactions pull the apical membrane into close association with the surface of the lipid sphere (Fig. 1.9c), allowing the sphere to migrate (bulge) towards the alveolar lumen and ultimately (by unknown mechanisms) to be pinched off (Jeong *et al.*, 2009; Mather, 2011a).

Xanthine oxidoreductase is an unusual partner for such a role. XOR is best known for its role in catalysis of the last two steps in the formation of uric acid, a nitrogenous waste product, but it has multiple enzymatic functions and is a member of the molybo-flavoenzyme (MFE) protein family (Garattini *et al.*, 2003). The MFEs are believed to have evolved as an ancestral XOR in prokaryotes, perhaps by the linkage of three genes that separately coded for what became the three distinctive domains of XOR, the 2Fe/2S, the FAD, and the MoCo domains (Garattini *et al.*, 2003). In mammals there are now four or five known MFEs, all of which share great structural and sequence similarity, and have apparently derived via tandem gene duplication from the ancestral XOR. Although the XOR gene is sometimes considered a housekeeping protein (Vorbach *et al.*, 2002), this is debatable as XOR is unequally expressed in cells and has particularly high expression in epithelial surfaces of the gastrointestinal tract, liver, kidney, lungs, skin, and mammary glands (Garattini *et al.*, 2003).

In mammals, XOR as initially synthesized has the characteristic binding sites and substrates of the enzyme xanthine dehydrogenase, and it is in this form that it is found in MFGM (Enroth *et al.*, 2000). However it can be converted via mild proteolysis or oxidation of sulfhydryls to the enzyme xanthine oxidase, which is the form typically recovered from milk (Enroth *et al.*, 2000; Nishino *et al.*, 2008). Xanthine oxidase generates free radical and reactive nitrogen species, and is upregulated during inflammation, leading to the hypotheses that XOR has important antimicrobial activities, perhaps even in milk (Martin *et al.*, 2004), and that XOR may have had an important role in the evolution of innate immunity (Vorbach, 2003). Certainly XOR and innate immunity are both of pre-vertebrate origin, and were important long before mammary glands evolved. Yet the

upregulation and apical membrane localization of XOR in mammary epithelial cells during mammary gland development (McManaman *et al.*, 2002), the binding of XOR to the B30.2 domain of butyrophilin (Jeong *et al.*, 2009), and the failure of milk fat globule formation in heterozygous XOR knockout mice (Vorbach *et al.*, 2002) all indicate a novel function for XOR in the MFGM (Fig. 1.9c). Given the antiquity of XOR and its long, conservative evolutionary history, adoption of this new function during mammary evolution must be considered a radical departure.

Secretion of milk fat droplets also involves adipophilin, a protein belonging to the PAT (for perilipin, adipophilin, and TIP47) family of proteins that play a role in stabilizing lipid droplets in a wide range of cells, including adipocytes, hepatocytes, macrophages, and lactocytes. The PATs are localized to the surface of lipid droplets (Fig. 1.9b) and among other functions may serve as gatekeepers restricting enzyme access to the lipids within (Brasaemle, 2007). The family is of ancient origin, being found in both vertebrates and invertebrates, and its members share an N-terminal domain (the PAT domain) as well as hydrophobic regions, a hydrophobic cleft near the C terminus (missing in perilipin A) and considerable identity among amino acid sequences (Miura *et al.*, 2002). In the mammary gland, adipophilin is expressed during the development of mammary epithelial cells in association with secretory differentiation and the accumulation of cytoplasmic lipid droplets; subsequently adipophilin is found in the budding MFG and in the MFGM in secreted milk (Russell *et al.*, 2007; Mather, 2011a). Microarray analysis of lactating mouse mammary glands indicate that adipophilin transcripts are among the most abundant transcripts, at a level comparable to caseins; moreover, adipophilin may play a role in facilitating lipid transfer from endoplasmic reticulum to cytoplasmic lipid droplets, in stimulating triglyceride synthesis, and in inhibiting lipolysis of lipid droplets (McManaman, 2009). Adipophilin appears to have a pre-mammary association with cytoplasmic lipid droplets, but its role is enhanced in the mammary gland, and it may play a role in formation of the MFGM, although this is not fully understood. In one model, adipophilin associated with the surface of the lipid sphere binds to XOR, stabilizing a butyrophilin/XOR/adipophilin complex (Fig. 1.9c) whereas in another model adipophilin via its hydrophilic cleft is directly bound to the bilayer membrane (Mather and Keenan, 1998; Mather, 2011b).

Thus at least three disparate proteins—one (butyrophilin)an apical surface protein apparently involved in immunity, the second (XOR) a cytosolic enzyme with multiple functions, and the third (adipophilin) a structural protein associated with cytoplasmic lipid droplets—appear to have developed new and/or enhanced functions in the coordinated secretion of milk fat globules . When in mammary evolution did this occur? Milk fat globule secretion presumably evolved from some prior form of fat secretion, perhaps by tetrapod or synapsid skin glands. Certainly some extant frogs secrete lipids as a means of reducing water loss across the skin (Lillywhite *et al.*, 1997; Lillywhite, 2006), and secreted lipids applied to eggs could have had an impact on egg moisture loss (Oftedal, 2002b). However, much more research is needed to understand the differences and similarities of secretory mechanisms among taxa and gland types.

Mammary glands bear developmental and structural resemblance to apocrine glands, which led to the hypothesis that mammary glands are derived from ancient apocrine-like glands (Oftedal, 2002a). One can imagine a scenario in which an ancestral apocrine secretion entailed the secretion of apical blebs containing cytoplasm, secretory vesicles and perhaps cytoplasmic lipid droplets, similar to the process described for some specialized apocrine glands such as human axillary apocrine glands, glands of Moll, ceruminous glands in the outer ear canal, and rodent Harderian glands (Gesase and Satoh, 2003; Stoeckelhuber *et al.*, 2003; Stoeckelhuber *et al.*, 2006; Stoeckelhuber *et al.*, 2011). It is known that ceruminous glands secrete lipid material by apical blebs. When the blebs disintegrate in the gland lumen, the various constituents are apparently released. A similar scenario has also been proposed for male reproductive tissues such as the epididymis and prostate gland (including

the rat anterior prostate or coagulating gland) that secrete particular proteins of cytoplasmic origin not via exocytosis of secretory vesicles but via apical blebbing, and the apical blebs themselves contain secretory vesicles (termed epididymosomes and prostasomes) (Wilhelm *et al.*, 1998; Auműller *et al.*, 1999; Dacheux *et al.*, 2005; Thimon *et al.*, 2008).

If the apocrine-like glands believed to be ancestral to mammary glands secreted in this manner, at least three steps would have been required to generate milk fat globules. First, increased transfer of glucose and fatty acids from circulation to the gland, and upregulation of lipid synthesis by the glandular cells (and perhaps downregulation of lipolysis) would be required for an increase in the density and/or size of adipophilin-coated cytoplasmic lipid droplets available for apical secretion. Second, amino acid substitutions of the cytoplasmic B30.2 domain of the transmembrane butyrophilin would enable the modified butyrophilin1A1 to bind to XOR, stabilizing the bleb membrane. Third, changes in structure and function of adipophilin and/or other proteins in the protein coat may have been required to permit closer association of the apical membranes to the lipid droplets and thereby exclude the majority of the cytoplasm during the blebbing process (Fig. 1.9c). These and other as yet unidentified transitions in the development, regulation, and structures associated with fat secretion would presumably have occurred stepwise over time, leading to an increased rate of secretion of apical blebs with progressively more lipid and less cytoplasm until ultimately the mammalian pattern of MFGs containing little cytoplasm (in the form of cytoplasmic crescents) was attained. In this scenario, fat secretion via MFGs would gradually replace the nutritional role of lipids provided by yolk, allowing the inactivation of vitellogenins involved in transport and storage of lipids in the egg yolk (Brawand *et al.*, 2008). This would presumably have had to occur prior to the miniaturization of the mammaliaforms in the late Triassic and Jurassic.

Further research is required on the detailed mechanisms involved in both apical bleb and milk fat globule secretion to determine if there are shared structural, developmental, regulatory and functional elements indicative of a shared origin, or if the apparent similarities are the result of convergent evolution among highly specialized glands all involved in transport of materials into a glandular lumen. Other similarities that have not been discussed herein, such as the congregation of secretory vesicles at the base of the protruding apical bleb and at the base of the protruding milk fat droplet (Wooding *et al.*, 1970; Metka and Nada, 1992; Gesase and Satoh, 2003), also warrant further investigation. It should be recognized, however, that apocrine secretion differs among tissues and glands. For example, the protrusion may be narrow (a bleb) or wide (an endpiece), and the separation of this bleb/endpiece may occur via pinching off (narrow blebs), or condensation and merging of exocytotic vesicles creating a gap (wide endpieces) or even via a line of demarcation involving new plasma membrane and cytoskeletal elements (tubules), with subsequent detachment (Gesase and Satoh, 2003). The latter two types of separation have been termed decapitation. Next to nothing is known about secretory mechanisms in the simple apocrine glands that secrete onto the skin surface in most mammals (Montagna and Parakkal, 1974) and that are thought most likely to resemble the ancestral apocrine-like glands (Oftedal, 2002a). Any attempt to compare apocrine secretory mechanisms to milk fat globule secretion must also take into account that the magnitude of secretion differs by many orders of magnitude, with apocrine gland secretions measured in μL while mammary gland secretions are measured in mL or L, depending on body size (Oftedal, 1984; Riek, 2011). Similarities in secretory constituents between apocrine secretion and mammary secretion such as mucins, lysozyme, lactoferrin, and defensins (Stoeckelhuber *et al.*, 2003; Stoeckelhuber *et al.*, 2006; Vorbach *et al.*, 2006) need not represent a recently shared evolutionary origin as these may simply reflect ancestral antimicrobial functions common to most if not all epithelial gland secretions.

1.6 Origin and Evolution of Milk Sugar Synthesis

All mammalian milks contain at least traces of sugar (Oftedal and Iverson, 1995); in most eutherians, the predominant sugar is lactose (galactose (β1-4) glucose), while in monotremes, marsupials and some eutherian carnivores oligosaccharides predominate, most of which contain a galactose (β1-4) glucose unit at the reducing end (Urashima *et al.*, 2001b; Messer and Urashima, 2002; Uemura *et al.*, 2009; Senda *et al.*, 2010). Both lactose and oligosaccharides with lactose at the reducing end appear to be unique to milk (e.g., Toba *et al.*, 1991), and required the development of a novel synthetic pathway in the evolving mammary gland or its antecedent apocrine-like gland. The milks that are devoid of galactose (β1-4) glucose, such as milks of sea lions and fur seals (family Otariidae), are also devoid of oligosaccharides (Urashima *et al.*, 2001a), indicating that lactose synthesis is an essential step in milk oligosaccharide synthesis.

The advantage of lactose, relative to glucose, is that it is a larger molecule and thus exerts less osmotic effect per unit mass, allowing more carbohydrate to be included in an isosmotic secretion such as milk. Lactose-based oligosaccharides continue this trend even further, such that marsupials (which secrete a preponderance of longer chain oligosaccharides) produce milks that can be 11–14% sugar at mid-lactation (Oftedal and Iverson, 1995). Milks containing lactose but little if any oligosaccharide (such as horse and zebra milks) do not exceed 7% sugar, while primate milks (which include both lactose and oligosaccharides) can reach 8–9% sugar (Oftedal and Iverson, 1995; Tilden and Oftedal, 1997; Urashima *et al.*, 2009; Goto *et al.*, 2010). Another advantage of lactose, which may have played a role in its evolution, is that as a novel sugar not found elsewhere in nature (Toba *et al.*, 1991), lactose is only available to bacteria that have evolved an ability to take it up and digest it, as occurs in *E. coli* and lactobacilli that upregulate the lac operon, expressing β-galactoside permease (to facilitate lactose uptake) and β-galactosidase (to hydrolyze lactose intracellularly). There is also evidence that beneficial bifidobacterial populations are able to utilize oligosaccharides that contain particular four-sugar sequences (Xiao *et al.*, 2010). Thus lactose, and lactose-based oligosaccharides, may have had a role in determining the microbial species that could colonize mammary secretion, whether in the mammary gland, on the surface of an egg, or in the digestive tracts of neonates.

The evolution of lactose synthesis is a remarkable example of a protein (or in this case two proteins) adopting a completely new function with minimal changes in structure. In the mammary secretory cell, the synthesis of lactose begins with the synthesis of a unique milk protein, α-lactalbumin, in the rough endoplasmic reticulum (Brew, 2003). α-Lactalbumin is then transported to the Golgi apparatus. A transmembrane protein in the trans-Golgi, β-1,4-galactosyltransferase 1 (β4Gal-T1), binds UDP-galactose, producing a conformational change that allows α-lactalbumin to be bound (Fig. 1.10) (Ramakrishnan and Qasba, 2001). When α-lactalbumin then binds to β4Gal-T1, it alters the specificity of β4Gal-T1, allowing glucose to become the acceptor sugar for galactose transfer, resulting in the synthesis of lactose. Thus α-lactalbumin acts as a regulator of β4Gal-T1, and without α-lactalbumin β4Gal-T1 does not synthesize lactose under physiological conditions (Brew, 2003). However β4Gal-T1 does have another function in the absence of α-lactalbumin, namely, the transfer of galactose from UDP-galactose to *N*-acetyl glucosamine at the terminus of N-linked oligosaccharide chains (Fig. 1.10a). Thus the original function of β4Gal-T1—and its current function in most mammalian cells—is the post-translational glycosylation of proteins via elongation of nascent oligosaccharide chains. The structure of the β4Gal-T1 (Fig. 1.10a) exhibits a three-dimensional conformation and cleft, and appropriately located amino acids, to favor the binding of oligosaccharides (rather than free monosaccharides) as sugar acceptors for the enzymatic transfer of galactose (Ramakrishnan *et al.*, 2002).

β4Gal-T1 is one of a family of β1,4-galactosyltransferases (β4Gal-T1 to T7) that serve to add galactose, from the donor UDP-galactose to various glycans in β1-4-linkage. For example, β4Gal-T1 is

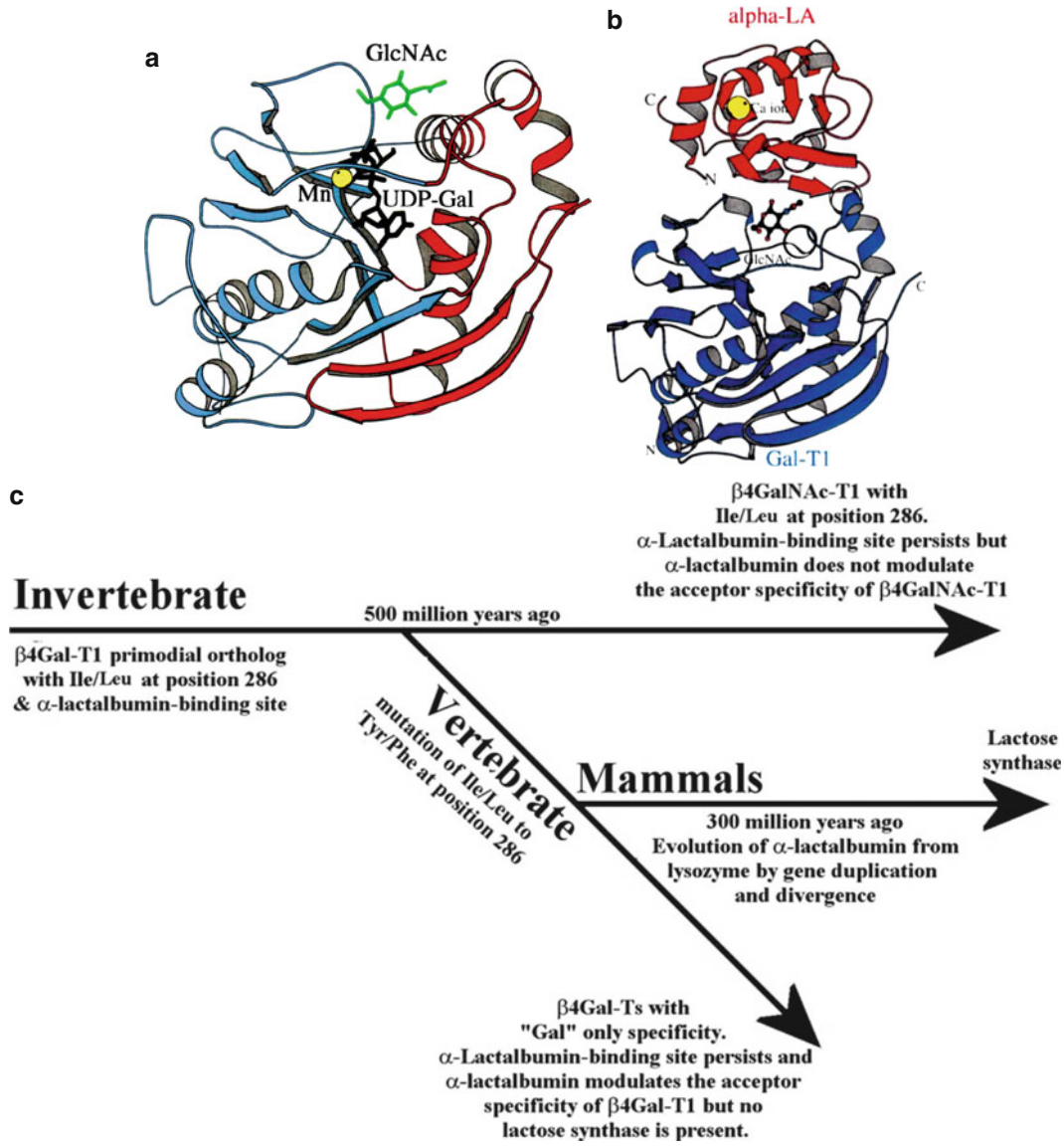

Fig. 1.10 Evolution of β4-galactosyltransferase-1 (β4Gal-T1) and lactose synthase. (**a**) Schematic structure (*side view*) of β4Gal-T1 showing acceptor site at top where bound UDP-galactose is ready to receive N-acetyl glucosamine, leading to addition of galactose to a protein-bound oligosaccharide chain. (**b**) Interaction of α-lactalbumin (above), but not c-lysozyme, with β4Gal-T1 (below) alters the sugar acceptor site so free glucose is preferentially accepted (not shown), leading to free lactose synthesis. (**c**) Hypothetical Ramakrishnan and Qasba (2007) model of evolution of β4Gal-T1. Due to amino acid substitution, invertebrate ortholog that transfers N-acetylgalactosamine is converted to β4Gal-T1 during early evolution of vertebrates, but the α-lactalbumin binding site had been previously established (although apparently nonfunctional). Once c-lysozyme evolves into α-lactalbumin about 200 million years later, lactose synthase activity arises, generating lactose (and/or lactose-based oligosaccharides; see text) (Credits: (**a**) Reproduced from Ramakrishnan *et al.* (2002), with copyright permission from Elsevier B.V.; (**b**) Reproduced from Ramakrishnan and Qasba (2001), with copyright permission from Elsevier B.V.; (**c**) Reproduced from Ramakrishnan and Qasba (2007), as corrected by P. Qasba, with copyright permission from Elsevier B.V.)

involved in the synthesis of the N-glycan of glycoproteins; β4Gal-T6 is involved in the synthesis of lactosylceramide, a building block for glycolipids; and β4Gal-T7 adds galactose to O-linked xylose on proteins during the synthesis of proteoglycans (Ramakrishnan and Qasba, 2007). These transferases are widespread in vertebrate tissues, and obviously predate the origin of mammals (Shaper *et al.*, 1998). Ramakrishnan and Qasba (2007) point out that β4Gal-transferases date back 500 mya to the invertebrate-vertebrate split (Fig. 1.10c), and apparently derived via amino acid substitution from β1,4-N-acetylgalactosaminyltransferase1 (β4GalNAc-T1), which is responsible for transfer of N-acetyl galactosamine rather than galactose. Remarkably, β4GalNAc-T1 in invertebrates has the ability to bind α-lactalbumin, indicating that this binding site long predates the origin of α-lactalbumin (Fig. 1.10c) (Ramakrishnan and Qasba, 2007).

No other β4Gal-transferase is currently known to bind to a regulator protein that modifies its specificity, and binding to a β4Gal-transferase is certainly not the original function of the protein that became α-lactalbumin. It has been apparent for many years, from amino acid sequence similarity, three-dimensional structure and the structure of the exons that code for α-lactalbumin, that α-lactalbumin is mostly closely related to c-type lysozyme and is derived from it via gene duplication and base pair substitution (Prager and Wilson, 1988; Qasba and Kumar, 1997; Brew, 2003). Lysozymes are hydrolytic enzymes that have the ability to cleave the β (1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer, and thus play a key role in innate immune defense systems in both vertebrates and invertebrates (Callewaert and Michiels, 2010). Amino acid substitutions have led to the loss of hydrolytic function in α-lactalbumin, so that it can no longer be considered a lysozyme even though this protein is derived from a lysozyme. The estimated date of origin of α-lactalbumin from c-lysozyme is ancient, prior to the time of the split of synapsids from sauropsids about 310 mya (Prager and Wilson, 1988).

Many authors have been puzzled by this date, as it was assumed that mammary glands did not arise until the appearance of "early mammals" (i.e., mammaliaforms) 100 million or more years later (Hayssen and Blackburn 1985; Prager and Wilson, 1988; Qasba and Kumar, 1997; Messer and Urashima, 2002). There has been speculation about some "intermediate function" that maintained the presence of this duplicated "lysozyme" prior to the evolution of lactose synthesis. One hypothesis was that the new protein retained lysozyme activity as it developed α-lactalbumin activity, as it was initially thought that monotreme α-lactalbumin retained some lysozyme activity. However, this has not been borne out by subsequent research (Messer and Urashima, 2002). It is worth noting that α-lactalbumin does not have lysozyme activity while lysozymes do not bind to β4Gal-T1, due to differences in amino acid composition at key positions involved in binding of substrate (in lysozyme) or binding of β4Gal-T1 (in α-lactalbumin) (Ramakrishnan and Qasba, 2001; Messer and Urashima, 2002; Brew, 2003; Callewaert and Michiels, 2010), so an "intermediate" with both functions may not have been possible.

It is probable that c-lysozyme, as a normal antimicrobial constituent of epithelial secretions and egg white (Callewaert and Michiels, 2010), would have been present in the earliest synapsid skin secretions, including secretions delivered to eggs; a c-type lysozyme is present in amphibian skin secretions (Zhao *et al.*, 2006). While the antimicrobial function of c-lysozyme would presumably help protect eggs (as it does in egg white), what advantage would accrue to eggs or hatchlings from the conversion of c-lysozyme function to that of α-lactalbumin, with the resultant synthesis of lactose? One must assume that lactose would have been indigestible to embryos or hatchlings given that the intestinal brush-border enzyme lactase could not have evolved without a substrate to digest, and lactose does not occur elsewhere.

Messer and Urashima (2002) argue that the ancestral function of α-lactalbumin as a β4Gal-T1 regulator may have been the production of

lactose-containing oligosaccharides, rather than free lactose per se. The amount of α-lactalbumin synthesized in the monotreme mammary gland is minor, and Messer and Urashima (2002) assume this to be the ancestral condition. It is intriguing that in some marsupials, such as the brushtail possum, lactose is the major sugar in both early (< 1 mo) and late (5–7 mo) milks, but oligosaccharides predominate in between (Crisp *et al.*, 1989a). In secreted brushtail possum milk the concentration of α-lactalbumin remains relatively constant over most of lactation (Grigor *et al.*, 1991). This implies that the expression and/or activity of trans-Golgi glycosyl transferases must change greatly over the course of lactation, being particularly high in the middle period.

A wide range of glycosyl transferases would have been present in the trans-Golgi of secretory cells of early synapsids as these are part of the normal synthetic machinery for glycosylation of glycoproteins, glycolipids, and proteoglycans in vertebrates and are of ancient origin (Fig. 1.10c) (Varki, 1998; Lowe and Varki, 1999). A low rate of lactose synthesis, coupled with high activity of glycosyl transferases that could glycosylate lactose, may have produced diverse oligosaccharides rather than free lactose, similar to what is observed in many extant monotremes and marsupials. In this scenario, the initial function of α-lactalbumin was as a step in the synthesis of free oligosaccharides, rather than the synthesis of free lactose. But why would the secretion of free oligosaccharides based on lactose be favored by natural selection? Milk oligosaccharides have antimicrobial or prebiotic effects, such as by leading pathogens to "mistake" free oligosaccharides for the oligosaccharide chains of the glycocaylx on apical cell membranes (Newburg, 1996) and thus to fail to bind to these surfaces. Such an effect might benefit the mammary gland, an egg surface, or the digestive tract of a hatchling even prior to the evolution of the lactase enzymatic mechanism in the young. It is intriguing that marsupial young that consume milks containing oligosaccharides but not lactose do not have intestinal lactase (Crisp *et al.*, 1989b), but whether this is the ancestral mammalian condition is not known. It is likely that the epithelium of the small intestine in monotremes and marsupials evolved the ability to take up milk oligosaccharides by endocytosis, followed by intracellular hydrolysis, but this has not been examined. If true, it may be the ancestral form of sugar digestion in synapsids and predate the evolution of lactase.

In eutherians, lactose accumulating in the Golgi apparatus creates an osmotic gradient which draws water into the Golgi, and this aqueous phase (including lactose, α-lactalbumin, other whey proteins, caseins, electrolytes) is packaged into secretory vesicles for transport to the apical plasma membrane of the secretory cell (Fig. 8a) (Shennan and Peaker, 2000). This model of milk secretion entails substantial upregulation of α-lactalbumin and lactose synthesis, and the transcription of β4Gal-T1 is also upregulated above constitutively expressed levels via use of a second transcriptional start site regulated by a stronger promoter and via more efficient translation of the truncated transcript (Shaper *et al.*, 1998). Although long considered the "standard" model of milk secretion, this may represent a derived feature of eutherian lactation that could only evolve after the young evolved the ability to digest lactose.

One group of eutherian mammals, the fur seals and sea lions (Pinnipedia: Otariidae), have secondarily lost the ability to synthesize α-lactalbumin, due to gene mutations and changes in transcription rates (Sharp *et al.*, 2005; Reich and Arnould, 2007; Sharp *et al.*, 2008), so the milk is devoid of lactose or lactose-based oligosaccharides (Oftedal *et al.*, 1987a; Urashima *et al.*, 2001a; Oftedal, 2011). The hypothesized advantage to these taxa is that the loss of α-lactalbumin eliminated an apoptotic signal (Sharp *et al.*, 2008), allowing these taxa to maintain functional lactocytes despite days or weeks without a suckling stimulus while the mothers are at sea feeding and the pups remain ashore (Oftedal *et al.*, 1987a). Lactose and/or α-lactalbumin are also reported as missing from Stejneger's beaked whale and the beluga whale (Ullrey *et al.*, 1984; Urashima *et al.*, 2002), although no selective advantage to this has been proposed. Otariids and cetaceans still manage to produce large volumes

of high-fat milk (Oftedal *et al.*, 1987b; Arnould and Boyd, 1995; Arnould *et al.*, 1996; Oftedal, 1997), but the secretory processes by which the aqueous phase is secreted have not been studied. In mice, knockout of the gene for α-lactalbumin results in a very low level of secretion of high-fat milk and the offspring do not survive (Stinnakre *et al.*, 1994; Stacey *et al.*, 1995).

As with casein and lipid secretion, if the early proto-lacteal secretions were minimal in volume, the constituents that were incorporated may have been rather different than those in milk as we know it today, such as a proto-casein rather than casein micelles, a small amount of fat-containing apical blebs rather than milk fat globules, and perhaps trace amounts of free oligosaccharides (as antimicrobial constituents) rather than large amounts of lactose in a voluminous aqueous phase. If early secretions served as sources of moisture and supplements for parchment-shelled eggs, the functions of these constituents may have been quite different from the major nutritional roles they play among extant mammals. The same may be true of some of the other major whey proteins.

1.7 Whey Proteins as Sources of Amino Acids

Milk proteins have been classically divided into caseins and whey proteins, with the latter remaining in solution when caseins are precipitated by enzymatic action or acid treatment. The major milk-specific whey proteins, depending on species, are α-lactalbumin, β-lactoglobulin, and whey acidic protein, but other primary whey proteins have been identified in marsupials, such as early lactation protein, late lactation protein, and trichosurin (Nicholas *et al.*, 1987; Piotte and Grigor, 1996; Demmer *et al.*, 1998; Piotte *et al.*, 1998). Whey proteins also include iron-binding proteins (such as lactoferrin and transferrin), serum albumin, immunoglobulins, various vitamin-binding proteins, and enzymes (including lysozyme) (Lönnerdal and Atkinson, 1995), but many of these proteins are imported from blood plasma rather than synthesized by the mammary gland and thus are not unique to milk. Investigations of the bovine lactation genome indicate that 3,111 genes are expressed in the mammary gland during lactation, but only a subset of these produce milk constituents and the functions of many of the expressed proteins are poorly understood (Lemay *et al.*, 2009).

Caseins have a loosely folded structure with few cystine disulfide bonds, and as a consequence contain a relative deficit of sulfur-containing amino acids (SAA, i.e., methionine and cysteine) relative to the requirements of offspring. In cow's milk, α_s-, β-, and κ-caseins contain about 2.9–3.7% SAA, by mass, whereas α-lactalbumin and β-lactoglobuin contain about 7–8% SAA (calculated from data presented in Fox (2003)). Suckling mammals appear to require that SAA represent 4–6% of total amino acids to attain maximal growth (Foldager *et al.*, 1977; Burns and Milner, 1981; Fuller *et al.*, 1989; National Research Council, 1995). Methionine can substitute for cysteine in most cases (except, perhaps, in premature human infants) (Fomon *et al.*, 1986; Thomas *et al.*, 2008), but cysteine can only replace about half of the methionine requirement in growing animals (Fuller *et al.*, 1989). In formulating casein-based diets supplemental cysteine or methionine are required to compensate for the SAA deficit in caseins (e.g., Reeves *et al.*, 1993; National Research Council, 1995). This suggests that other proteins had to coevolve with caseins if milk was to become a balanced source of amino acids, rather than just a supplement. The major milk-specific whey proteins, depending on species, are α-lactalbumin (e.g., in human milk), β-lactoglobulin (e.g., in cow's milk), and whey acidic protein (e.g., in rat milk).

1.8 Origin and Evolution of β-Lactoglobulin

The major whey protein in most ruminant milks (including dairy animals such as dairy cattle, goats, sheep, and water buffalo), β-lactoglobulin, does have not any indisputable biological role beyond supplying amino acids to the offspring (Sawyer, 2003). As β-lactoglobulin occurs in the milks of monotremes (platypus), several marsupi-

als (brushtail possum, wallabies, and kangaroos), and at least 35 species of eutherians, it must have evolved prior to the divergence of these groups in the Jurassic or Cretaceous.

The discovery that β-lactoglobulin was similar in structure to retinol-binding protein (RBP) led to the hypothesis that β-lactoglobulin might have a role in the transport of vitamin A, vitamin D, fatty acids, or some other essential lipophilic compounds to the young, or play a role in intestinal uptake of these constituents (Pervaiz and Brew, 1985; Perez and Calvo, 1995; Yang *et al.*, 2009). However, in ruminants vitamin A is associated with the milk fat globule, not with β-lactoglobulin, and in pigs and horses β-lactoglobulin does not bind either retinol or fatty acids. In genetically modified mice, β-lactoglobulin may assist in vitamin D absorption (Yang *et al.*, 2009), but mouse milk normally lacks β-lactoglobulin and if pups required it for vitamin D uptake they would develop vitamin D deficiencies. Thus, if β-lactoglobulin in some cases plays a role in transport and/or intestinal uptake of these constituents, it is neither essential nor universal (Perez and Calvo, 1995). Another problem in ascribing a functional role is that β-lactoglobulin is absent in the milks of many mammals, including laboratory mice and rats, guinea pigs, domestic rabbits, dromedary camels, llamas, and humans (Sawyer, 2003). It is often stated that rodent milks lack β-lactoglobulin but this is based on only three of more than 2,200 rodent species, and thus is not certain. Among primates, human milk lacks β-lactoglobulin, but it is present in the milks of at least three macaque species and the hamadryas baboon (Hall *et al.*, 2001).

Both β-lactoglobulin and RPB are members of a large family of small extracellular proteins, termed lipocalins, that have similar tertiary structure, specific amino acid sequence motifs and exon-intron structure of coding genes (Flower, 1996; Åkerstrom *et al.*, 2006). This ancient protein family (or superfamily) apparently derives from a bacterial protein and is characterized by a barrel-shaped lipophilic cavern surrounded by a series of 8 β-strands and open on one end (Fig. 1.11a) (Ganfornina *et al.*, 2006). Many lipocalins are known to function via transport and/or sequestration of hydrophobic compounds in this "barrel" and occasionally at secondary binding sites. Such ligand binding is associated with a wide diversity of functions (Åkerstrom *et al.*, 2006), including as anticoagulants and anti-inflammatory agents (e.g., by binding of histamine, serotonin, and other molecules by lipocalins in tick and spider saliva), as vehicles for color enhancement and retention (e.g., by binding of carotenoids, biliverdin, and other pigments by lipocalins in arthropod epidermis), as components of antimicrobial defense (e.g., by sequestration of bacterial siderophores or liberated heme groups by lipocalins in vertebrate fluids), as fat-soluble vitamin transport (e.g., by binding of retinol by a lipocalin in vertebrate extracellular fluids), and even as nutrient provision to offspring (e.g., by proposed binding of cholesterol by "Milk proteins" in cockroach epidermal secretions) (Williford *et al.*, 2004).

Analysis of the molecular evolution of the lipocalins provides some information about the origin of β-lactoglobulin. In chordates the lipocalins have been classified into 12 clades (Fig. 1.11b,c), all of which are found in mammals (Ganfornina *et al.*, 2000; Sanchez *et al.*, 2003; Sanchez *et al.*, 2006). Clades I and II are apolipoproteins D and M which resemble invertebrate lipocalins and probably originated prior to the evolution of chordates. Among the chordate lipocalins the RBPs (clade III) appear to occupy a basal position (Fig. 1.11c), leading to the hypothesis that most vertebrate lipocalins evolved from a RBP-like lipocalin (Sanchez *et al.*, 2006). In a phylogenetic tree of the lipocalin family, the β-lactoglobulins (clade IV) are the sister group to clades V–XII, which nest together (Sanchez *et al.*, 2006). This suggests that RBP diverged first, followed by β-lactoglobulin; subsequently clades V to XII diverged from each other (Fig. 1.11c). If this is correct, β-lactoglobulin (clade IV) is of more ancient origin than clades V and VI which are found in fish and amphibians (Fig. 1.11b). It appears that the ancestral β-lactoglobulin gene may have appeared prior to amniotes and perhaps even prior to tetrapods. Unfortunately, the great extent of nucleotide and amino acid substitution that is characteristic of lipocalins (Flower, 1996)

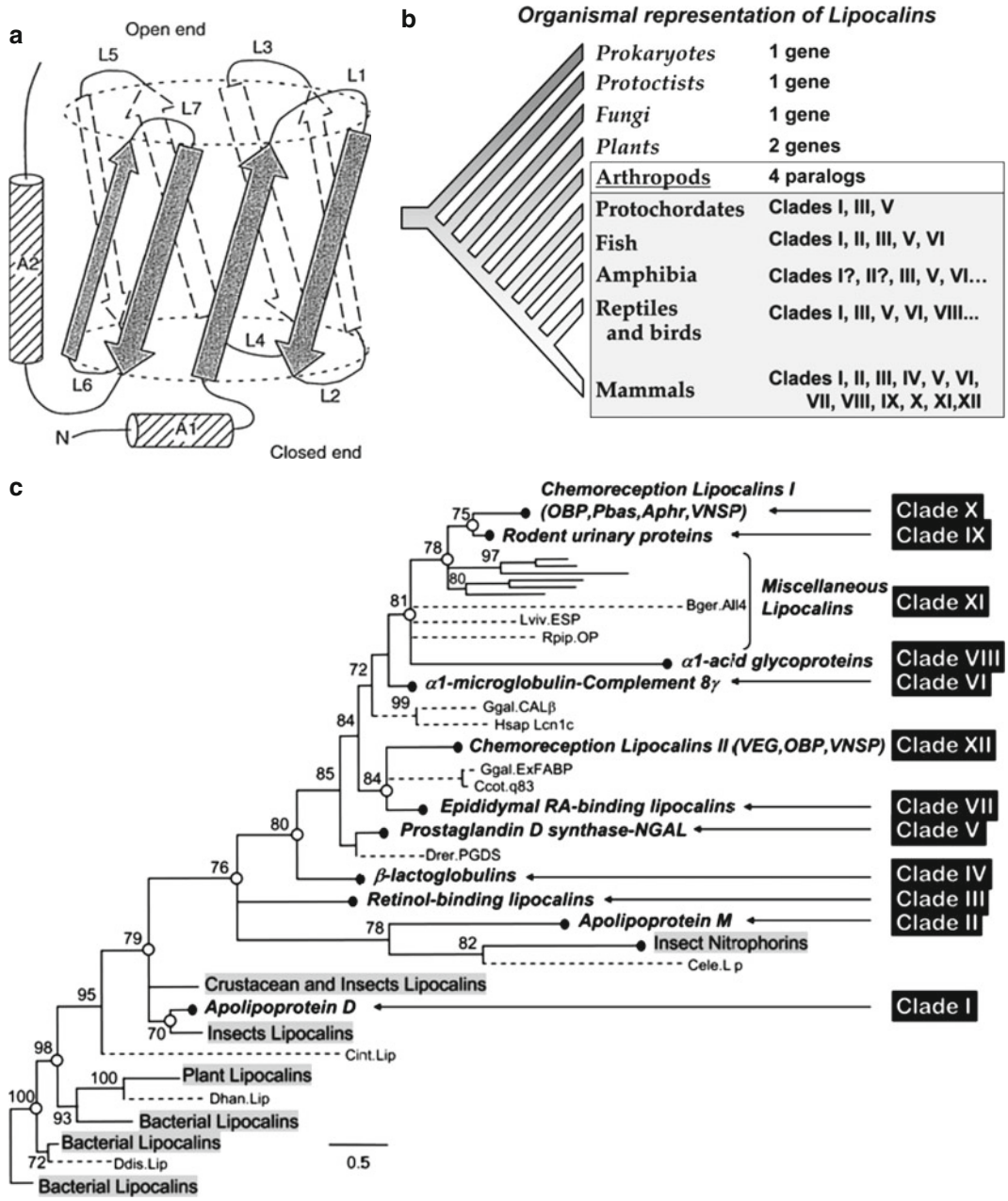

Fig. 1.11 The evolution of lipocalins including β-lactoglobulins. (**a**) The structures of lipocalins include a folding motif that involves an eight-stranded antiparallel β-barrel (*broad arrows*) connected by loops (L1-L7) and an α-helix at both the N-terminal and C-terminal ends (A1 and A2, respectively). The barrel is open at one side and encloses a binding pocket; lipocalins also have an ability to form oligomers, from dimers to octamers. (**b**) Taxonomic distribution of lipocalin clades. β-Lactoglobulins represent clade IV. (**c**) Phylogenetic consensus tree of the lipocalin family from protein sequences, reconstructed by a Bayesian method, and rooted with bacterial lipocalins. Posterior clade probability values (>70) are shown at each node. The *scale bar* represents the branch length (number of amino acid substitutions/site) (Credits: (**a**) Reproduced from Ganfornina *et al.* (2000), with copyright permission from Oxford University Press ; (**b**, **c**). Reproduced from Sanchez *et al.* (2006), with copyright permission from Landes Bioscience)

would probably obscure the genetic remnants of a β-lactoglobulin pseudogene in the genomes of non-mammalian vertebrates.

It is likely that the ancestral β-lactoglobulin had a function similar to that of an ancestral RBP-like protein, that is, transporting hydrophobic compounds in extracellular and/or secreted fluids, long before the appearance of milk as we know it. Given the wide variety of functions associated with extant lipocalins, it is possible that an ancestral β-lactoglobulin served multiple functions, which by analogy to other constituents that evolved into milk proteins, might include antimicrobial defense in the proto-lacteal gland, on the skin surface, or on the surface of eggs, and was later co-opted into the function of amino acid provision to the young. An intriguing parallel has been proposed in the case of another type of lipocalin (the "Milk proteins") in cockroaches. In live-bearing cockroaches "Milk proteins" secreted by surface epithelial cells have been co-opted from initial ligand transport functions into a nutritive role for developing offspring in the brood pouch (Williford *et al.*, 2004).

Although β-lactoglobulin retains a generalized ability to bind a variety of hydrophobic ligands, due to the elasticity of the outer parts of the barrel (Konuma *et al.*, 2007), its role in milk appears to be primarily a nutritional one as a source of amino acids (and particularly limiting sulfur amino acids). In some taxa, such as the domestic dog, cat, horse and ass, and perhaps the bottlenose dolphin, β-lactoglobulins are expressed from two or three genes (Ganfornina *et al.*, 2000; Sawyer, 2003). In species in which other whey proteins predominate, β-lactoglobulin can become superfluous and the ability to synthesize it can be lost. Thus in rats, mice, and guinea pigs, whey acidic protein is dominant, while in humans α-lactalbumin predominates, both of which are as high or higher in SAA than β-lactoglobulin. Two β-lactoglobulin genes have been observed in ruminants, but one is noncoding and is thus a pseudogene (Sawyer, 2003). A β-lactoglobulin pseudogene is also suspected in the human genome, but there may be confusion with the glycodelin gene (Kontopidis *et al.*, 2004). Additional lipocalins (trichosurin, late lactation protein) are expressed in marsupial milk (Demmer *et al.*, 1998; Piotte *et al.*, 1998), but these are only distantly related to β-lactoglobulin and are apparently of more recent origin (Ganfornina *et al.*, 2000).

It has recently been suggested (Kontopidis *et al.*, 2004) that β-lactoglobulin may derive from another lipocalin, glycodelin (previously known as human β-lactoglobulin homolog, placental protein 14, progestogen-dependent endometrial protein, pregnancy-associated endometrial α2-globulin, and progesterone-associated endometrial protein). Although also found in rats (Kunert-Keil *et al.*, 2005), glycodelin is best known in humans, where it is secreted by various tissues into amniotic, follicular, uterine, and seminal fluids, including the glandular and luminal surface of the endometrium; it also has limited expression in bone marrow, non-lactating mammary tissue, and other tissues (Seppälä, 2002; Seppälä *et al.*, 2006, 2007). A major role of glycodelin is in protection of reproductive products (the sperm, zygote, implantation site, and developing embryo) from maternal immune responses. Glycodelin has been demonstrated to suppress lymphocyte proliferation, induce phenotypic change in dendritic cells, inhibit T and B cell activity and proliferation, and induce apoptosis in monocytes (Seppälä *et al.*, 2009). Glycodelin expression into fallopian and uterine secretions is under hormonal regulation and is upregulated during time periods suitable for fertilization and implantation (Seppälä *et al.*, 2009). Based on nucleotide sequence similarity, glycodelin is nested within the β-lactoglobulins in lipocalin clade IV (Fig. 1.11c) (Ganfornina *et al.*, 2000). Given this similarity, (Kontopidis *et al.*, 2004) proposed that glycodelin may be ancestral to β-lactoglobulin: "Might it be that the protein glycodelin reflects the true, original function of β-LG as a protein involved in some aspect of fetal development in all mammals?" Or as Cavaggioni *et al.* (2006) put it: "it is certainly possible that BLG has arisen as the result of a gene duplication event from an essential, possibly endometrial, lipocalin, such as glycodelin, with a probable transport function crucial for the development of the endometrium during the early stages of pregnancy."

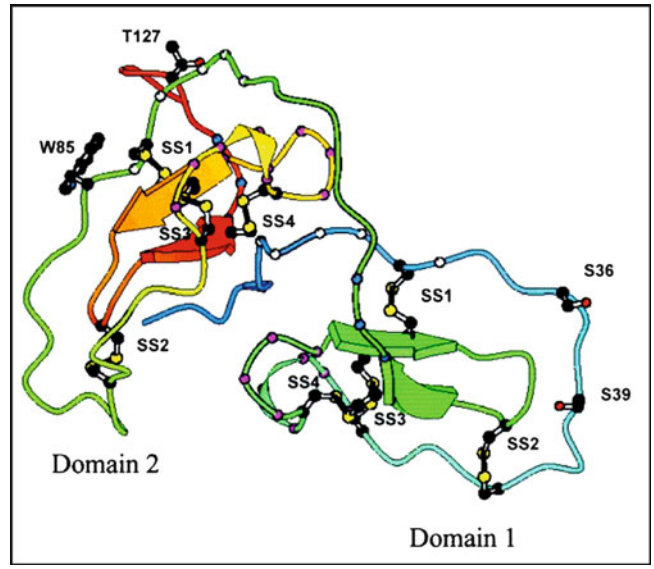

Fig. 1.12 Whey acidic protein (WAP) in milk. Structural model of a eutherian WAP (from pig milk) including two WFDC domains each of which contains four disulfide bridges between cysteine pairs (SS1–SS4). Putative glycosylation sites (serine, S36 and S39, and threonine, T127) and a conserved tryptophan (W85) are also illustrated (Credit: Reproduced from Ranganathan *et al.* (1999), with copyright permission from Elsevier B.V.)

There are several problems with this hypothesis. First is that lactation—and β-lactoglobulin—long preceded the origin of the mammalian uterus, including the uterine role in endometrial secretions, implantation of the blastocyst, and nutrient transport via a placenta (Finn, 1998; Oftedal, 2002a). For example, β-lactoglobulin is found in milk of the platypus, which has a secretory oviduct that is considered a forerunner of the eutherian uterus (Finn, 1998). Second, glycodelin is a very unusual lipocalin in that it is not only highly glycosylated, but the isoforms generated by different tissues differ in function according to their tissue-specific glycosylation patterns (Seppälä *et al.*, 2007). Third, glycodelin does not bind retinol, fatty acids, or other potential hydrophobic ligands that have been tested (Seppälä *et al.*, 2006), even though most β-lactoglobulins do. Thus glycodelin is a highly derived lipocalin that has acquired structure (N-linked oligosaccharides) and lost function (hydrophobic ligand binding) in taking on a new and specialized role. These derived features are not found in β-lactoglobulin. While it seems impossible that an ancestral glycodelin-like protein in endometrium could have evolved into β-lactoglobulin, the converse—that the glycodelin gene derives from an ancestral β-lactoglobulin gene—is certainly possible.

1.9 Origin and Evolution of Whey Acidic Protein

Whey acidic protein (WAP) is a whey protein present in representatives of all three major mammalian lineages—monotremes, marsupials, and eutherians—indicating that WAP is pre-mammalian in origin (Sharp *et al.*, 2007). The key feature of WAP is the presence of two or three domains of about 40–50 amino acids, each of which contains 8 cysteine residues involved in four disulfide bonds (Fig. 1.12); as the domain was first recognized in WAP, it was initially known as the WAP domain and more recently as the Whey Acidic Protein Four-Disulphide Core (WFDC) domain. In this chapter I refer to the milk protein as whey acidic protein or WAP, and the domain as the WFDC domain, to avoid confusion between the two. WAPs are only a subset of the WFDC-containing proteins. Among whey proteins, WAP has the highest sulfur amino acid content, about 17–20% by mass, and thus represents an excellent source of sulfur amino acids for suckling young.

There are at least 33 distinct (non-homologous) proteins among vertebrates and invertebrates that include 1–4 WFDC domains (see PROSITE, www.expasy.org/cgi-bin/prosite), including proteins with antibacterial, antiviral,

and anti-inflammatory functions, as well as several proteinase inhibitors. All are secreted proteins including proteins in respiratory, reproductive, and other epithelial secretions (Hagiwara *et al.*, 2003; Bingle *et al.*, 2006). The structural similarity of WAP to other WFDC-containing proteins has led to speculation that WAP may also have antibacterial or proteinase inhibition functions, but attempts to demonstrate this have failed (Hajjoubi *et al.*, 2006; Sharp *et al.*, 2007). Recently Bingle and Vyakarnam (2008) reviewed the structure and function of 18 human WFDC proteins. The amino acid sequence of the WFDC1 protein (also termed PI3, coded on human chromosome 16) is highly conserved across taxa (from zebra fish to chicken to mouse to man), suggesting a close relationship between structure and function that has been maintained by purifying selection. In contrast, many of the 14 WFDC proteins coded at the WFDC locus on human chromosome 20 (including WFDC2) exhibit high levels of amino acid substitution even among closely related taxa, such as primates (Hurle *et al.*, 2007; Bingle and Vyakarnam, 2008). In comparing amino acid sequences among human WFDC proteins, Bingle and Vyakarnam (2008) conclude that all non-cysteines are substituted in one WFDC domain or another, and suggest that the spacing of the cysteines involved in disulfide bridges may more important for function than the actual identities of amino acids in the inter-cysteine regions. The high rate of amino acid substitution also complicates efforts to determine evolutionary relationships, since amino acids may have been gained and lost multiple times without leaving a record of these transitions.

The WFDC domain itself is of ancient origin, being a component in secreted proteins involved in the regulation of shell mineralization in mollusks such as abalone (Treccani *et al.*, 2006) and in antimicrobial response as part of the innate immunity of crustaceans and perhaps insects (Zou *et al.*, 2007; Jia *et al.*, 2008; Smith *et al.*, 2010b). A number of WFDC domain-containing proteins are also secreted by snake venom glands, where they have antibacterial function (Nair *et al.*, 2007; Fry *et al.*, 2008), and by skin glands in frogs where they serve as antimicrobial defensive compounds (Ali *et al.*, 2002; Zhang *et al.*, 2009). While much more research is required to determine relationships among invertebrate and vertebrate WFDC-containing proteins, it is likely that an ancestral WAP present in the glandular skin secretion of an egg-tending tetrapod or early synapsid served as a defensive compound against microbes as a component of the innate immune system, similar to existing WFDC proteins in mammalian epididymal, respiratory, and oral mucosal secretions (Hiemstra, 2002; Hagiwara *et al.*, 2003; Bingle and Vyakarnam, 2008) and in frog skin secretions (Ali *et al.*, 2002; Zhang *et al.*, 2009).

There is evidence that WFDC domains influence cell proliferation and growth in vitro and in transgenic mice (reviewed by (Topcic *et al.*, 2009)), but when the WAP gene is deleted in knockout mice, the mice continue to develop normal mammary glands. This indicates that WAP is not essential for mammary cell differentiation or proliferation even though WAP is commonly used as a marker of mammary cell differentiation in mouse cell culture (Triplett *et al.*, 2005). The primary effect of WAP deletion in mice appears to be growth retardation of the young during the second half of lactation. This appears to be a consequence of maternal factors, not neonatal genotype, as pup performance was not influenced by pup genotype (Triplett *et al.*, 2005). The absence of milk WAP (as assessed by SDS-polyacrylamide gel electrophoresis) and a likely reduction in milk yield (as suggested from mammary histology) in these knockout mice indicate a reduction in supply of sulfur amino acids to mouse pups during a period of active growth of the body and pelage (which is high in sulfur amino acids). It would be interesting to know if mouse pups from $WAP^{-/-}$ mothers show clinical or biochemical signs of sulfur amino acid deficiency; Triplett *et al.* (2005) did not observe reduced glutathione levels in splenocytes and thymocytes of growth-retarded pups, but other indicators were not examined.

An intriguing feature of the WAPs is that protein size differs among taxa, associated with differing numbers of WFDC domains. Marsupial and platypus WAPs have three WFDC domains,

while eutherian and echidna WAPs have only two WFDC domains (Sharp *et al.*, 2007). It has been suggested that the ancestral WAP had three (Demmer *et al.*, 2001) or four (Sharp *et al.*, 2007) domains, and that the two domains found among eutherians are a consequence of loss of ancestral domains. The different domains have been assigned to domain groups based on similarity of amino acid sequence. Thus even taxa with the same numbers of WFDC domains in WAP may not have the same domains. The two domains in eutherians were originally named domains I and II, but the third domain discovered in marsupials (domain III) was found at the N-terminal end, so the domain order in marsupials is domain III–domain I–domain II (Simpson *et al.*, 2000; Demmer *et al.*, 2001). However, according to sequence comparisons, neither of the monotremes has domain I, but rather have domain III plus one or two copies of domain II (in the echidna: domain III–domain IIa; in the platypus: domain III–domain IIa–domain IIb) (Sharp *et al.*, 2007).

Using cluster analysis, (Sharp *et al.*, 2007) conclude that the domains II of eutherians and marsupials are most similar to monotreme domains IIa and IIb, respectively. It was this observation of two different types of domain II that led to the hypothesis that the ancestral WAP may have had four WFDC domains in the order DIII-DI-DIIa-DIIb, and that exon loss may have resulted in loss of different domains within different mammalian lineages (Sharp *et al.*, 2007). This hypothesis is tentative, however, because the high degree of amino acid substitution and insertion, especially between the first 3 cysteines at the N-terminal end of the WFDC domain, may include reversals or multiple changes, muddying the phylogenetic signal. Moreover, there is only moderate bootstrap support for some of the branches in the consensus cluster diagrams of Demmer *et al.* (2001) and Sharp *et al.* (2007), suggesting that additional data may lead to different consensus clusters. The purported four-domain structure of the ancestral WAP protein is also unusual, as most extant WFDC proteins have only one or two WFDC domains, and other than monotreme and marsupial WAP, even three domain WFDC proteins are uncommon (see PROSITE, www.expasy.org/cgi-bin/prosite).

As with β-lactoglobulin, there is evidence that WAPs in various milks may have lost function, at least among eutherian mammals. First, WAP genes appear to have been lost in some taxa. Although the genes for WAP synthesis have been found in sheep, goats, and cattle, they are missing a nucleotide at the end of the first exon, causing a frameshift mutation (Hajjoubi *et al.*, 2006). They are not transcribed and are thus pseudogenes. The WAP gene is expressed in both pigs and camels, which represent lineages that diverged early from other artiodactyl lineages, so the ancestral condition in this order was apparently to secrete WAP in milk. There is no current evidence that WAP is secreted by primates, but the presence of a putative pseudogene in the human genome (Hajjoubi *et al.*, 2006) suggests primate ancestors secreted WAP; a functional WAP gene may yet be found when the milk and mammary genes of more primates (including strepsirrhines) are examined. Second, even among eutherians that secrete WAP, there is evidence that amino acid substitutions may have disrupted WFDC domain structure, possibly altering or interfering with function. In WFDC proteins that exhibit protease inhibition the three-dimensional configuration, including an external loop and antiparallel β-strands linked by cystine disulfide bridges (Fig. 1.12), appears to be critical to interactions with the protease (Ranganathan *et al.*, 1999). Yet in WFDC domains in both mouse WAP (domain I) and rabbit WAP (domain II) 1–2 cysteines have been lost, with loss of disulfide bridging, raising questions about functionality (Sharp *et al.*, 2007). Third, of the two WFDC domains in WAP in eutherian milks, designated as DI and DII, only DII retains the characteristic N-terminal motif found in most WFDC domains (Lys-X-Gly-X-Cys-Pro, where X represents various amino acids); amino acid substitutions in this and other areas of DI may have altered the charge distribution, glycosylation sites, and conformation in such a way that original functions are no longer possible (Ranganathan *et al.*, 1999).

The apparent degradation of eutherian WAPs raises the question of whether monotreme and marsupial milks retain functional WAPs that have importance because of the extreme immaturity of their offspring (Sharp *et al.*, 2007). WAP expression varies with lactation stage in these taxa, suggesting some functional role, but more evidence is needed (Topcic *et al.*, 2009). An initial speculation that WAP might have protease inhibition function that could protect milk immunoglobulins from degradation has not been confirmed. As with β-lactoglobulin, the only certain role of WAP is as a source of sulfur amino acids for suckling young.

1.10 Conclusion: Patterns in Milk Protein Evolution

The fact that lactation is so ancient, stemming back in time to early synapsids in the Carboniferous (or in proto-lacteal form, perhaps even to the more ancient tetrapods), means that there has been ample opportunity for evolutionary change in the structure and functions of secreted proteins. The caseins diversified via gene duplication and exon changes long before mammals evolved, and were transformed from simple SCPPs engaged in mineral regulation to highly complex micelles bearing responsibility for amino acid, calcium, and phosphorus transport to rapidly growing young. Although this must have been achieved before the late Triassic, when mammaliaforms were miniaturized by evolution and the ontogeny of dentition was delayed (i.e., diphyodonty), the ongoing need for nutritional investment in the young has preserved casein genes, in some cases in multiple copies. Milk fat globule proteins (butyrophilin1A1, XOR, adipophilin) were co-opted from roles in innate immunity and cytoplasmic fat droplet synthesis into new functions associated with the MFGM. At or before the appearance of synapsids, a c-lysozyme was duplicated and subsequently structurally modified in a way that permitted it to transition from an antimicrobial constituent to a new role regulating the preferred acceptor for a galactosyl transferase, and thereby became involved in secretion of a suite of lactose-based sugars. Two other whey proteins, β-lactoglobulin and WAP, may also have had ancestral transport, sequestration, or antimicrobial functions in skin secretions, but appear to have lost these functions and now serve primarily or solely as supplemental sources of amino acids, and especially sulfur amino acids.

It appears that there was an early experimentation with secretory constituents that converted them from roles in biochemical transport and regulation, and in defense against potential pathogens, at epithelial and/or egg surfaces to new functions related to the nutritional needs of offspring. The early experimentation may have begun among Carboniferous tetrapods that had a highly glandular skin, or among early synapsids in the Carboniferous or Permian, but it must have been well advanced prior to the progressive rise of metabolic and growth rates, and miniaturization of adult size, that occurred during the late Triassic, or the appearance of mammals in the Jurassic. Note that this represents an extremely long period of time (Fig. 1.2), nearly 200 million years from the beginning of the Carboniferous to the appearance of mammals about 160 mya. Thus milk and the proto-lacteal secretion that preceded it may have a combined evolutionary history that is more than twice as long as that of mammals per se. Lactation was no doubt one of the many mammal-like traits repeatedly modified during the sequential radiations of synapsids in the Carboniferous, Permian, Triassic, and Jurassic.

Today we regard mammary glands and lactation as unique mammalian accomplishments, but that is because all of the other pre-mammalian synapsid lineages went extinct, leaving mammals as the only surviving synapsids, and thus the sole surviving milk-dependent group. Given the antiquity of lactation, it is not surprising that some milk constituents—including β-lactoglobulin and WAP—appear to be relicts of once-functional proteins that are now important primarily because of their amino acid composition. However, the loss of ancestral functions should not be taken to mean that milk constituents are unimportant: on the contrary, comparative genomic analysis reveals that the genes involved in milk synthesis

are highly conserved across mammals as compared to genes coding for other tissues and functions (Lemay *et al.*, 2009). There may be tremendous variation across mammalian taxa in the relative proportions of different milk constituents, correlated to differences in patterns of maternal care, constraints on maternal physiology, physiologic requirements of suckling young, and other life history phenomena (Oftedal, 1984, 1993; Oftedal and Iverson, 1995; Oftedal, 1997; Tilden and Oftedal, 1997; Oftedal, 2000, 2011), but the fundamental pattern of mammary secretion, once acquired over the ages, appears to have been largely conserved since the late Triassic.

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Milk Proteins: Introduction and Historical Aspects

2

J.A. O'Mahony and P.F. Fox

2.1 Introduction

Milk is a fluid secreted by the female of all mammals, of which there are about 4,500 species, primarily to meet the complete nutritional requirements of the neonate. The principal requirements are for energy (supplied by lipids and lactose and, when in excess, by proteins), essential amino acids and amino groups for the biosynthesis of non-essential amino acids (both supplied by proteins), essential fatty acids, vitamins, inorganic elements and other minor nutritional factors, such as taurine, and for water. Because the nutritional requirements of the neonate depend on its maturity at birth, its growth rate and its energy requirements, which depend mainly on environmental temperature, the gross composition of milk shows large interspecies differences, which reflect these requirements. The gross composition of the milk of a number of species is shown in Table 2.1. How well the milk protein system meets the nutritional requirements for protein is discussed in Chap. 17.

Milk also serves a number of other physiological functions, most of which are served by proteins and peptides. The physiologically important proteins and peptides include immunoglobulins, enzymes, enzyme inhibitors, growth factors, hormones and antibacterial agents. These aspects are discussed in Chaps. 9, 10, 11 and 12.

In addition to meeting the nutritional and other requirements of their own neonates, the milk of certain domesticated animals, and dairy products produced therefrom, are major components of the human diet in many parts of the world. Domesticated goats, sheep and cattle have been used for milk production since about 8000 BC. Recorded milk production today is about 589×10^6 tonnes per annum, about 85% of which is bovine, 11% is buffalo and about 2% each is ovine and caprine, with small amounts produced from horses, camels, donkeys, yaks and reindeer. In many European countries, the USA, Canada, Australia and New Zealand, about 30% of dietary protein is supplied by milk and dairy products.

As a dietary item, milk has many attractive features:

- Nutritionally, it is the most complete single food available.
- It is free from toxins and anti-nutritional factors.
- It has a pleasant and attractive flavour and mouthfeel.

However, the ease with which milk can be converted to a wide range (several thousand) of different and attractive products is probably its most important feature from an industrial viewpoint. The manufacture of many of these products relies on some rather unique properties of milk proteins, which have, therefore, attracted considerable research attention. This research is

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Table 2.1 Composition (%) of milk of some species (Fox, 2003)

Species	Total solids	Fat	Protein	Lactose	Ash
Human	12.2	3.8	1.0	7.0	0.2
Cow	12.7	4.5	2.9	4.1	0.8
Sheep	19.3	7.4	4.5	4.8	1.0
Pig	18.8	6.8	4.8	5.5	–
Horse	11.2	1.9	2.5	6.2	0.5
Donkey	11.7	1.4	2.0	7.4	0.5
Reindeer	33.1	16.9	11.5	2.8	–
Domestic rabbit	32.8	18.3	11.9	2.1	1.8
Bison	14.6	3.5	4.5	5.1	0.8
Indian elephant	31.9	11.6	4.9	4.7	0.7
Polar bear	47.6	33.1	10.9	0.3	1.4
Grey seal	67.7	53.1	11.2	0.7	–

facilitated by the ease with which the proteins can be isolated from milk.

Today, milk proteins are probably the best characterized of all food proteins. The current status of knowledge on milk proteins will be described in the following chapters. The objective of this chapter is to provide a brief history and overview of research on milk proteins to help link later chapters into a more coherent body of information.

Research on milk proteins dates from 1814, when the first paper on the subject was published by J.J. Berzelius (1814). The term casein appears to have been first used in around 1830 by H. Braconnot who developed a method for the preparation of protein from milk by acid precipitation. The term 'protein' was coined by J.G. Mulder in 1838, whose work included studies on casein. Early researchers were confused as to the nature of proteins. They believed that there were three types of protein: albumin (e.g., egg white and blood serum), fibrin (muscle) and casein (milk curd), each of which occurred in both animals and plants (Johnson, 1868). The caseins were then thought to be those plant or animal proteins that could be precipitated by acid or by calcium or magnesium salts.

The acid (isoelectric) precipitation of casein was refined by O. Hammersten during the period 1883–1885, and, consequently, isoelectric casein was for a long time referred to as 'casein *nach* Hammersten'. Initial chemical analysis of casein showed that it was a unique protein, the properties of which differed from those of other proteins known at that time. In around 1880, Danilewsky and Radenhausen proposed that acid-precipitated casein is heterogeneous, but this was refuted by Hammersten who claimed that properly prepared isoelectric casein is homogeneous. In 1890, Halliburton proposed that the term caseinogen should be used for the acid-insoluble protein in milk which was converted to casein by the action of rennet (the suffix 'ogen' meaning to beget). About 70 years ago, the term 'casein' was universally adopted as the English word for the protein precipitated from milk at pH 4.6. Casein is converted by rennet to *para*casein (i.e. 'like casein'; using the techniques available at that time, it was not possible to differentiate between casein before and after rennet action), which is coagulated by Ca^{2+}. Based on differential solubility in ethanolic solutions, evidence began to emerge from the work of Osborne and Wakeman in 1918 and of Linderstrøm-Lang and collaborators during the period 1925–1929 that isoelectric casein is a heterogeneous protein. Heterogeneity was confirmed by the application of the analytical ultracentrifuge by Pedersen in 1936 and of free-boundary electrophoresis by Mellander in 1939 to the study of caseins (see McMeekin, 1970 for references to early literature). These techniques resolved casein into three components, namely, α, β and γ in order of decreasing electrophoretic mobility.

The liquid remaining after isoelectric precipitation of casein from skimmed or whole milk is called whey. It is a dilute solution of proteins, referred to as whey or serum proteins, which are present at a concentration of approximately ~0.7% in bovine milk, lactose, inorganic salts, vitamins and several other constituents are present at trace levels. In 1857, Bouchardat and Quevenne showed that milk contains an albumin (initially termed 'lactalbumin'). Using salting-out with $MgSO_4$, the whey proteins were fractionated by Seblein in 1885 into soluble (albumin) and insoluble (globulin) fractions. In 1899, A. Wichmann crystallized a protein from the albumin fraction

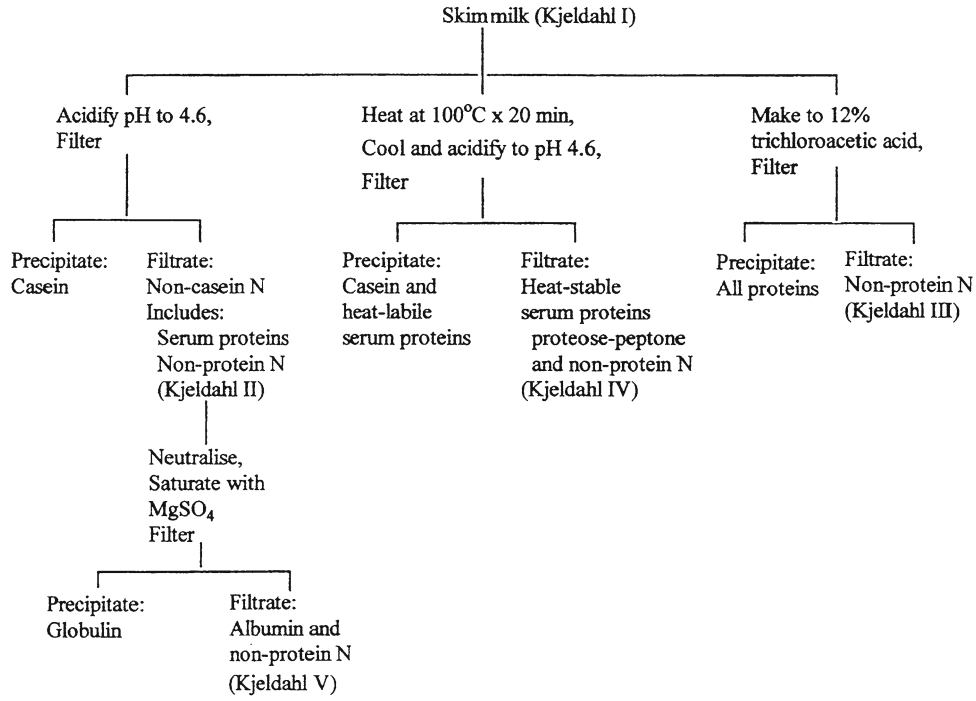


Fig. 2.1 Classical fractions of milk proteins (Fox, 2003)

of whey by addition of $(NH_4)_2SO_4$ and acidification, a technique which was used at that time to crystallize blood serum albumin (BSA) and ovalbumin. Using the techniques available at the end of the nineteenth century, the whey proteins were found to be generally similar to the corresponding fractions of blood proteins and were considered to have passed directly from blood to milk; consequently, the whey proteins attracted little research attention until the 1930s (see Sect. 2.9).

In addition to the caseins and whey proteins, milk contains two other groups of proteinaceous materials: (1) proteose peptones (PPs) and (2) non-protein nitrogen (NPN). These fractions were recognized by Rowland (1938) who observed that when milk was heated at 95°C for 10 min, ~80% of the whey proteins were denatured and co-precipitated with the caseins when the heated milk was acidified to pH 4.6. He concluded that the heat-denaturable whey proteins were the lactoglobulins and lactalbumins and that the remaining 20% represented a different protein(s), which he designated 'proteose peptone'. The PP was precipitated by 12% trichloroacetic acid (TCA), but some nitrogenous compounds remained soluble in 12% TCA, which were designated NPN. Rowland described a scheme (Fig. 2.1) for the fractionation and quantitation of the major groups of milk proteins. This scheme, which was modified by Aschaffenburg and Drewry (1959), is still used widely to quantify the principal protein groups in milk to provide information for protein quality and processing. The NPN has low or no commercial value, and the proteose peptones are not recovered in cheese or casein.

Thus, by 1938, the general complexity of the milk protein system had been described (i.e. caseins, lactalbumin, lactoglobulin, PP and NPN) which represents approximately 78%, 12%, 5%, 2% and 3%, respectively, of the nitrogen in bovine milk. However, at this stage, knowledge of the milk protein system was very rudimentary and vague, as is evident from perusal of such texts as *Richmond's Dairy Chemistry* (Davis

and MacDonald, 1953), *A Textbook of Dairy Chemistry* (Ling, 1944) and *Fundamentals of Dairy Science* (Associates of L. A. Rogers, 1935) or earlier editions of these books.

Knowledge on the chemistry of milk proteins has advanced steadily during the twentieth century and can be followed through the progression of textbooks on Dairy Chemistry (Jenness and Patton, 1959; Webb and Johnson, 1965; McKenzie, 1970, 1971a; Webb *et al.*, 1974; Fox, 1982, 1989, 1992; Walstra and Jenness, 1984; Barth and Schlimme, 1988; Wong *et al.*, 1988; Jensen, 1995; Cayot and Lorient, 1998; Fox and McSweeney, 1998; Walstra *et al.*, 1999, 2005). In addition, there have been numerous monographs and reviews, of which the following are particularly useful from a historical viewpoint: Eilers *et al.* (1947), McMeekin and Polis (1949), Pyne (1955), Jenness *et al.* (1956), Brunner *et al.* (1960), Lindqvist (1963), Thompson *et al.* (1965), Jolles (1966), McKenzie (1967), Rose *et al.* (1970), Lyster (1972), Swaisgood (1973), Whitney *et al.* (1976), Brunner (1981), Eigel *et al.* (1984), Kinsella (1985), Kinsella and Whitehead (1989), Holt (1992), Wong *et al.* (1996), Farrell *et al.* (2004) and Fox and Brodkorb (2008).

2.2 Preparation of Casein and Whey Proteins

Although isoelectric precipitation is the most widely used method for separating the casein and non-casein fractions of milk protein, several other techniques may be used in certain situations and are described below. The protein fractions may be prepared from whole or skimmed milk, but the latter is almost always used as the fat is occluded in the protein precipitate produced by many methods and will interfere with further characterization of the proteins. The fat is removed easily by centrifugation, e.g., 3,000 × *g* for 30 min, and any remaining fat may be removed by washing the precipitated protein with ether. In the following, 'milk' refers to skimmed milk, unless stated otherwise.

2.2.1 Isoelectric Precipitation of Casein

On reducing the pH of milk to ~4.6, the caseins aggregate and, if acidified under quiescent conditions, form a coagulum. Aggregation occurs at all temperatures, but <~6°C, the aggregates are very fine and remain in suspension but can be sedimented by low-speed centrifugation. At higher temperatures (30–35°C), the aggregates are coarse and precipitate readily from solution. Above ~45°C, the precipitate tends to be stringy and difficult to handle.

In the laboratory, HCl is usually used for acidification, although lactic or acetic acid may be used also. Industrially, HCl is also the most widely used acidulant. Lactic acid produced in situ by a culture of lactic acid bacteria (LAB) is used also, especially in New Zealand, the principal producer of industrial casein. In milk, the casein occurs as coarse colloidal particles (micelles; see Chap. 6), which include calcium phosphate and other salts, collectively referred to as colloidal calcium phosphate (CCP). When milk is acidified, the CCP dissolves and is completely solubilised <pH 4.9. If sufficient time is allowed for equilibrium to occur, isoelectric casein is essentially free from calcium phosphate. Best results are obtained by acidifying the milk to pH 4.6 at ~4°C, holding for at least 30 min and then warming to ~35°C. The fine aggregates formed at 4°C allow time for the CCP to dissolve; a moderately dilute acid (~1 M) is preferred since a concentrated acid may cause localized precipitation. After holding at 35°C for ~30 min, the whey is removed by filtration through cheesecloth or other suitable material, and the casein is washed thoroughly by repeated suspension in distilled water, followed by filtration; thorough washing is essential for the removal of lactose and salts. Some investigators prefer to dilute the milk with water before acidification in order to obtain a finer precipitate, with less inclusion of other compounds. Removal of impurities may also be effected by washing the casein curd by dispersing it in water and raising and maintaining the pH at ~7 by addition of NaOH or other alkali

and re-precipitation by acid. The casein may be stored frozen or dried by washing with acetone or freeze-drying.

Unlike bovine milk, for human and equine milks, the pH at which casein and whey proteins are optimally separated by isoelectric precipitation is not pH 4.6. Casein in equine milk displays minimum solubility at pH 4.2 (Uniacke-Lowe and Fox, 2011), while caseins and whey proteins in human milk are typically separated at pH 4.3 (Kunz and Lonnerdal, 1992). Due to the poor curd-forming properties of human milk (Lonnerdal and Forsum, 1985), in addition to acidification, such milk may also be supplemented with calcium to enhance curd formation in the separation of casein from whey proteins.

2.2.2 Ultracentrifugation

The casein micelles are quite large (molecular weight [MW] ~ 10^8–10^9 Da), and, consequently, most (90–95%) of the casein in milk is sedimented by centrifugation at 100,000 × *g* for 1 h. Sedimentation is more complete at 35°C than at 0–4°C; as at low temperature, some of the casein (in particular β-casein) dissociates from the micelles (Rose, 1968) and is therefore non-sedimentable by ultracentrifugation. The whey proteins, which are molecularly dispersed or present as small oligomers, are not sedimentable and remain in the supernatant. Casein prepared by ultracentrifugation contains the original level of CCP and can be re-dispersed in a suitable buffer as micelles with properties similar to those of the original micelles. Casein micelles prepared in this way are very useful for the study of their properties in the absence of whey proteins.

2.2.3 Centrifugation After Enrichment with Calcium

Addition of $CaCl_2$ to ~0.2 M causes aggregation of the casein to such an extent that it can be sedimented readily by low-speed centrifugation. If Ca-fortified milk is heated to ~90°C, the casein aggregates and precipitates without centrifugation. The whey proteins are denatured on heating at 90°C and co-precipitate with the caseins to yield a product known as casein-whey protein co-precipitate, which is processed in a manner similar to that used for casein. Casein co-precipitates are produced on a commercial scale but have enjoyed only limited commercial success, with poor solubility being a significant limitation to their use in many applications. This method is not useful for the production of casein for research purposes.

2.2.4 Salting-Out Methods

Casein can be precipitated from solution by any of several salts. Addition of $(NH_4)_2SO_4$ to milk at a concentration of 260 g L^{-1} causes complete precipitation of the caseins, together with some of the whey proteins (immunoglobulins [Igs]). Saturation of milk with $MgSO_4$ or NaCl may be used also; again, the Igs co-precipitate with the caseins. Saturated NaCl gives clean fractionation of the caseins and most of the whey proteins, provided that they are undenatured, and is used to separate caseins, Igs and denatured lactalbumins from undenatured whey proteins for the heat classification of milk powders. It has been argued (see McKenzie, 1971b) that salting-out methods cause less denaturation than isoelectric precipitation, but the latter is almost always used to separate caseins from the whey proteins.

2.2.5 Membrane Filtration

All the milk proteins are retained by small-pore, semi-permeable membranes that may be used to isolate the total proteins from milk or the whey proteins from whey; the proteins are in the retentate while lactose, soluble salts and other small molecules are in the permeate. This process, referred to as ultrafiltration, is used widely for the industrial-scale production of whey protein concentrates (WPCs) and to a lesser extent for the production of milk protein concentrates (MPCs). Intermediate pore size (i.e. 0.1–1.0 μm) microfiltration membranes are used to a limited

extent industrially to separate casein micelles from whey proteins. The casein fraction produced using such technology is referred to as phosphocasein, native micellar casein or milk casein concentrate, while the whey fraction is referred to as native, ideal or virgin whey (Pierre *et al.*, 1992; Kelly *et al.*, 2000; Rizvi and Brandsma, 2002). These casein- and whey-enriched ingredients have many interesting and high growth potential applications in the areas of cheese milk fortification, infant nutrition, clinical nutrition and premium physico-chemical functionality applications (e.g., high gel strength). Microfiltration membranes with even larger pores (1–2 μm) are used to remove bacteria, spores and other particulate matter from milk, with both casein and whey proteins being present in the permeate. This technology is used to remove microorganisms from milk (>99.9% removal) for the production of extended shelf-life beverage milk and cheese milk. It is also used to remove lipoprotein particles from whey in the production of defatted WPC and whey protein isolates (WPIs).

2.2.6 Gel Filtration

It is possible to separate the caseins from the whey proteins by gel permeation chromatography on Sephadex or other suitable medium, but this method is not used either on a laboratory or industrial scale. It is also possible to resolve the individual whey proteins by gel permeation, which is used to a limited extent for laboratory-scale preparation of protein fractions and for analytical methods involving whey protein profiling, quantification and assessment of denaturation (Wang and Lucey, 2003; Roufik *et al.*, 2005; Kehoe *et al.*, 2007; Liskova *et al.*, 2010).

2.2.7 Precipitation by Ethanol

The caseins may be precipitated from milk by ~40% ethanol, while the whey proteins remain soluble; lower concentrations of ethanol may be used at lower pH values. The caseins appear to be precipitated in micellar form and may be re-dispersed in water or buffer (Hewedi *et al.*, 1985). Much of the fundamental information on ethanol stability of milk was generated by David Horne and co-workers at the Hannah Research Institute over 20 years ago (see Horne, 2003), and while precipitation by ethanol does not appear to be used either on a laboratory or industrial scale for the preparation of casein, this work has provided a basis for understanding and optimizing the stability of cream liqueur products.

2.2.8 Cryoprecipitation

Caseins, in a mainly micellar form, may be destabilized and precipitated by freezing milk or, preferably, concentrated milk at about −10°C. Precipitation is caused by a decrease in pH and an increase in Ca^{2+} concentration arising from the precipitation of soluble $CaHPO_4$ and $Ca(H_2PO_4)_2$ as colloidal $Ca_3(PO_4)_2$, with the release of H^+; the decrease in pH causes an increase in Ca^{2+} concentration. Cryoprecipitated casein is reported to have good solubility and curd-forming properties, which may be advantageous, compared with alternative methods for the production of casein on a laboratory scale; however, it is reported to have inferior emulsifying properties compared with sodium and calcium caseinates (Moon *et al.*, 1988, 1989). To the authors' knowledge, cryoprecipitated casein is not being produced commercially.

2.2.9 Rennet Coagulation

The casein micelles are destabilized by specific, limited proteolysis and precipitate or coagulate in the presence of Ca^{2+}. The casein thus precipitated is altered, and its properties are very different from those of isoelectric casein (Mulvihill and Ennis, 2003). Some properties of rennet casein make it very suitable for certain food applications (e.g., analogue cheese manufacture) (Ennis and Mulvihill, 1999; O'Sullivan and Mulvihill, 2001).

2.2.10 Preparation of Caseinates

Isoelectric casein, and some of the other forms of casein prepared as described above, is insoluble in water but may be converted to water-soluble caseinates by dispersion in water and adjusting the pH to ~6.7 with alkali, usually NaOH to yield sodium caseinate. KOH, NH_4OH or $Ca(OH)_2$ can also be used, giving the corresponding caseinate (Mulvihill and Ennis, 2003). In the laboratory, caseinates may be freeze-dried but are usually spray-dried in industrial-scale production.

2.2.11 Preparation of Whey Proteins

Although the methods described above are focused on the preparation of casein, the whey proteins are, obviously, obtained as a second stream and may be prepared from the whey obtained in any of the above procedures by salting-out or by removing the non-protein constituents by dialysis, crystallization and/or ultrafiltration. However, some whey proteins are co-precipitated with the caseins by some of the methods, and rennet whey contains casein-derived peptides (e.g., glycomacropeptide) liberated by the rennet. On laboratory scale, the whey protein-enriched streams prepared using the above approaches are typically freeze-dried for further analysis.

2.3 Comparison of Key Properties of Casein and Whey Proteins

2.3.1 Solubility at pH 4.6

As described above, the caseins are, by definition, insoluble at pH 4.6, whereas the whey proteins are soluble under the ionic conditions of milk, although they are least soluble around pH 4.6, with isoelectric points ranging from approximately pH 4.2 to 5.4 (Gordon, 1971; McKenzie, 1971d). The isoelectric precipitation of casein is of major industrial significance since it permits the production of caseins and caseinates, fermented milk products and acid-coagulated cheeses.

2.3.2 Coagulability by Limited Proteolysis

Also, as described above, the caseins are coagulable after specific, limited proteolysis, whereas the whey proteins are not. This property of the caseins is exploited in the production of the rennet-coagulated cheese (~75% of all cheese) and rennet casein.

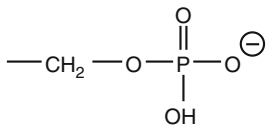
2.3.3 Heat Stability

The caseins are very heat-stable. Milk at pH 6.7 may be heated at 100°C for 24 h without coagulation and withstands heating at 140°C for up to 20–25 min; aqueous solutions of sodium caseinate are even more stable and may be heated at 140°C for several hours without apparent changes. The heat stability of the whey proteins is typical of globular proteins, and they are denatured completely on heating at 90°C for 10 min. The remarkably high heat stability of the caseins, which is probably due to their lack of typical stable secondary and tertiary structures (see Chap. 5), permits the production of heat-sterilized dairy products with relatively small physical changes. The heat stability of milk (especially in concentrated systems) is very important in the manufacture of many commercial milk-based products and will be discussed in more detail in Volume 1, Part B.

2.3.4 Amino Acid Composition

The amino acid composition of the individual milk proteins will be discussed in the appropriate chapters. Suffice it to state here that the caseins contain high levels of proline (17% of all residues in β-casein) which largely explains their lack of α-helix and β-sheet secondary structures. All the caseins are phosphorylated, while the principal whey proteins are not. Whole isoelectric casein contains ~0.8% phosphorus, but the degree of phosphorylation varies among the individual caseins (see Chap. 4). The phosphate moieties

are attached to the caseins mainly as phosphomonoesters of the serine side chain:



The presence of phosphate groups has major significance for the properties of the caseins, e.g.,:

- Molecular charge and related properties such as hydration, solubility and possibly heat stability are affected by the presence of phosphate groups.
- Metal binding is strongly affected; most of the calcium, zinc and inorganic phosphorus in milk are associated with the caseins and affect their physico-chemical, functional and nutritional properties. It has been suggested (Holt, 1994) that the metal-binding properties of casein might be regarded as a biological function since they enable a high concentration of calcium phosphate to be carried in milk in a soluble form (to supply the requirements of the neonate); otherwise, calcium phosphate would precipitate in and block the ducts of the mammary gland.
- As a consequence of metal binding, usually of Ca^{2+}, most of the caseins are precipitated by polyvalent cations, which may be desirable or undesirable, depending on the product; it is essential for the rennet coagulation of milk, as in cheese manufacture.
- The caseins contain a low level of sulphur (0.8%), while the whey proteins are relatively rich (1.7%). Differences in sulphur content are more apparent when the individual sulphur-containing amino acids are considered. The sulphur of casein occurs mainly in methionine, with little cysteine; in fact, the principal casein is devoid of this amino acid. The whey proteins are relatively rich in cysteine, which has major effects on their properties and on the physico-chemical properties of milk; these effects will be discussed in several later chapters.
- The whey protein α-lactalbumin is relatively rich in tryptophan, which, due to its role in synthesis of the neurotransmitter serotonin, is important for the use of α-lactalbumin-enriched whey protein ingredients in infant and clinical nutritional products.

2.3.5 Site of Biosynthesis

The caseins are synthesized in the mammary gland and are unique to this organ. Presumably, they are synthesized to meet the amino acid requirements of the neonate and, as indicated above, as carriers of important elements required by the neonate. The principal whey proteins are also synthesized in, and are unique to, the mammary gland, but several minor proteins in milk are derived from blood, either by selective transport or due to leakage. Most of the whey proteins have a biological function, which will be discussed in the appropriate chapters.

2.3.6 Physical State in Milk

The whey proteins exist in milk as monomers or as small quaternary structures. In contrast, the caseins exist as large colloidal aggregates, known as micelles. The micelles in bovine milk range from ~50 to 500 nm in diameter, with an average of ~150 nm and an average molecular mass of ~10^8 Da and contain about 5,000 molecules. The white colour of milk is due largely to the scattering of light by the casein micelles. The caseins in the milk of different species occur as micelles, at least all are white but the micelles of only ~15 species have been examined (Buchheim *et al.*, 1989). They range in size from ~50 nm in human milk to ~500 nm in equine and asinine milks. The structure, properties and stability of the casein micelles are of major significance for the technological properties of milk and, consequently, have been the subject of intensive research which is reviewed later in this chapter and in Chap. 6.

2.4 Heterogeneity and Fractionation of Casein

Hammersten and subsequent workers for the next 40 years believed that well-prepared isoelectric casein was a homogeneous protein. However, during the early years of the twentieth century, some evidence was presented that it might be heterogeneous, which was first demonstrated by

Osborne and Wakeman and by Linderstrøm-Lang and collaborators (see McMeekin, 1970). By treatment of isoelectric casein with ethanol-HCl mixtures, Linderstrøm-Lang and Kodoma (1929) obtained three major casein fractions, which differed considerably in phosphorus content, about 1.0, 0.6 and 0.1%, and several minor fractions. However, it was suggested that the rather severe fractionation method used by these workers may have caused artefacts, and the heterogeneity of casein was not generally accepted until the application of analytical ultracentrifugation by Pedersen (1936) and free-boundary electrophoresis by Mellander (1939) to the study of casein. Electrophoresis, which was performed under mild conditions, showed clearly that isoelectric casein is a mixture of three proteins, which were named α, β and γ in order of decreasing electrophoretic mobility; these proteins represented about 75%, 22% and 3% of whole casein, respectively. Following the demonstration of its heterogeneity, several attempts were made to fractionate casein into its components. The first reasonably successful method was that of Warner (1944), who exploited differences in the solubility of α- and β-caseins at pH 4.4 (the isoelectric point of α-casein) and 2°C; under these conditions, β-casein (isoelectric pH ~4.9) is more soluble than α-casein. Repeated precipitation and resolubilization under these conditions gave reasonably homogeneous preparations of α- and β-caseins, but yields were low, and the method was time-consuming. A much more satisfactory fractionation method was developed by Hipp *et al.* (1952). In fact, these workers developed two methods based on (1) differential solubilities of α-, β- and γ-caseins in urea solutions at pH 4.9 or (2) on differential solubility in ethanol-water mixtures. The urea method is easier and more effective and was widely used for many years until the widespread application of ion-exchange chromatography. The use of a high concentration of urea has been criticized because it causes extensive denaturation of proteins, which is not particularly serious in the case of caseins, which are not highly structured. In addition, urea decomposes to ammonium carbamate and ammonia, especially at alkaline pH, and on heating, carbamate reacts with the ε-group of lysine to form homocitrulline.

The phosphorus content of the α-, β- and γ-caseins isolated by Hipp *et al.* (1952) was 1.0%, 0.6% and 0.1%, respectively, i.e. similar to the values reported by Linderstrøm-Lang and Kodoma (1929) for their three major fractions. The α-casein peak which tended to split on free-boundary electrophoresis (Tobias *et al.*, 1952; Slatter and van Winkle, 1952) was shown by Waugh and von Hipple (1956) to consist of two proteins with very different properties, which were referred to as α_s- and κ-caseins. Although it was well known that caseinates were precipitated by Ca^{2+}, the possibility of using Ca^{2+} to fractionate the caseins was not reported until that time. Waugh and von Hipple (1956) sedimented the casein micelles from Ca-enriched (0.6 M) milk and re-dispersed the pellet (which contained all the caseins and a little whey protein) in 0.4 M potassium oxalate which sequestered micellar calcium (citrate was later usually used for this purpose). The insoluble calcium oxalate formed was removed by centrifugation and excess soluble oxalate removed by dialysis. The resulting protein solution was very similar to Na-caseinate and was referred to as *first cycle casein.* It is not clear why ultracentrifugally prepared micelles rather than Na-caseinate were used to prepare first cycle casein. When first cycle casein at 27°C was made to 0.25 M with $CaCl_2$, part of the protein precipitated but part remained soluble. The Ca-insoluble fraction, referred to as *second cycle casein*, was shown by free-boundary electrophoresis to be mainly α- and β-caseins while the soluble fraction, referred to as *fraction S*, was found to contain β-casein and a heretofore unknown protein, which was called κ-casein. The Ca-insoluble α-casein was called α_s-casein, 's' signifying Ca-sensitive (Fig. 2.2).

κ-Casein, which represents ~15% of total casein, is soluble in the presence of Ca^{2+} and when mixed with the calcium-sensitive α_s- and β-caseins, can stabilize them against Ca^{2+} in milk with the formation and stabilization of casein micelles, in which it serves as the protective colloid (*schutz* colloid). κ-Casein is hydrolysed by rennet resulting in the coagulation of milk. It is

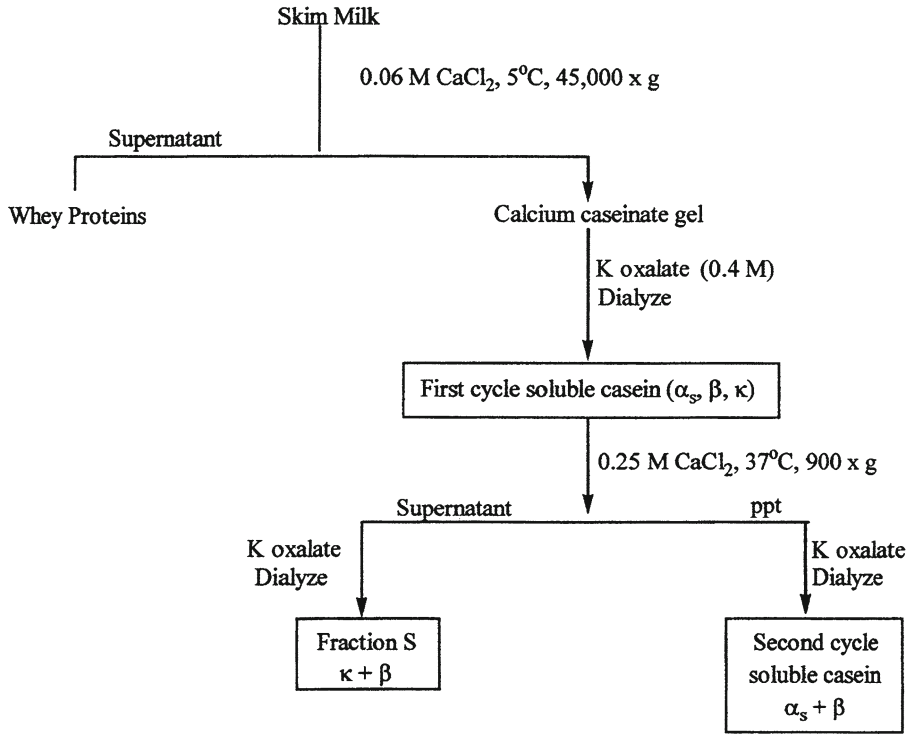


Fig. 2.2 Fractionation of casein according to Waugh and von Hipple (1956)

also responsible for many other technologically important properties of the milk protein system. Numerous chemical methods were soon developed for the isolation of κ-casein, probably the most widely used of which were those of Swaisgood and Brunner (1962) and Zittle and Custer (1963), both of which use very severe conditions—12% TCA or pH 1.3–5, respectively, in 6.6 M urea.

Prepared by the method of Waugh and von Hipple (1956), α_s-casein was found to be very heterogeneous when analyzed by gel electrophoresis. Apart from contamination with β- and κ-caseins, it can be resolved into one major, two medium and several minor bands. These components were resolved by Annan and Manson (1969) and named α_{s0} to α_{s6}. It is now known that these proteins are of two distinctly different types, now named α_{s1}- and α_{s2}-caseins, each of which is heterogeneous. The cause of the heterogeneity is explained below under Sect. 2.6.

The methods of Hipp *et al.* (1952) and of Zittle and Custer (1963) were combined by Fox and Guiney (1972) to provide a method for the preparation of α_{s1}-, α_{s2}-, β-, κ- and γ-caseins in relatively large quantities and in fairly homogeneous forms. However, chemical methods have now been largely superseded by ion-exchange chromatography, which gives superior results, although on a smaller scale. For research and analytical purposes, the caseins are usually fractionated by anion-exchange chromatography, usually using a buffer containing a reducing agent (usually 2-mercaptoethanol) and a high concentration (5–6 M) of urea to reduce and dissociate the caseins, respectively (see Strange *et al.*, 1992; Imafidon *et al.*, 1997). More recently, chromatographic methodology has also been developed for simultaneous identification and quantification of the major casein and whey proteins in milk-based products (Bordin *et al.*, 2001); samples are treated with guanidine hydrochloride, dithiothreitol and trisodium citrate before resolution on a C_4 reversed phase HPLC column, with photodiode array detection.

Strategies for fractionation and enrichment of individual caseins, suitable for scale-up, have

also been developed at laboratory and pilot scale (Murphy and Fox, 1991; Huppertz *et al.*, 2006; O'Mahony *et al.*, 2007). Such approaches primarily exploit the tendency for β-casein to dissociate from casein micelles in milk/aqueous caseinate dispersions at low temperature with centrifugal separation or membrane filtration used to harvest the β-casein-enriched supernatant or permeate fractions, respectively. β-Casein is reported to be more easily digested than whole casein or α_s-casein, which is of particular significance for infant and clinical nutrition applications. To the authors' knowledge, the only commercially available β-casein-enriched product is that manufactured by Kerry Ingredients, Tralee, Co. Kerry, Ireland (Ultranor Beta™).

2.5 Application of Gel Electrophoresis to the Study of Milk Proteins

Zone electrophoresis on solid media was introduced in the 1940s. Initially, filter paper and later cellulose acetate were used and gave good results with many protein systems. However, since the caseins have a very strong tendency to associate hydrophobically, the resolution obtained by electrophoresis on paper or cellulose acetate was little better than that obtained with free-boundary electrophoresis, although easier to operate. Electrophoresis on starch gels (SGE) which was introduced to general protein chemistry in 1955 (Smithies, 1955; Poulik, 1957) was applied to the study of the caseins by Wake and Baldwin (1961). The resolving power of SGE was far superior to that of any of its predecessors. Wake and Baldwin (1961) used a discontinuous tris-citrate/borate buffer and included 7 M urea in the gels to dissociate the caseins. The method was improved (Neelin, 1964) by including the reducing agent, 2-mercaptoethanol, in the starch gel to reduce the intermolecular disulfide bonds in α_{s2}- and κ-caseins; this modification resulted in several discrete bands for κ-casein which otherwise formed a smear. SGE resolved isoelectric casein into about 20 bands which were shown to be due to the microheterogeneity of the principal caseins, as described in Sect. 2.5. The high degree of heterogeneity shown by SGE in casein indicated the need for a rational nomenclature system; both Wake and Baldwin (1961) and Neelin (1964) proposed nomenclature systems; the system proposed by the former was widely adopted but has been extensively modified as the cause of the heterogeneity of casein became clear (see Sect. 2.5).

Electrophoresis on polyacrylamide gels (PAGE) was applied to the study of the caseins by Peterson (1963). PAGE and SGE give similar results, but PAGE is far easier to use and has become the standard electrophoretic method for analysis of caseins (and most other protein systems). Gel electrophoretic methods for the analysis of milk proteins have been reviewed by Swaisgood (1975), Strange *et al.* (1992), Van Hekken and Thompson (1992), O'Donnell *et al.* (2004) and Chevalier (2011a, b). The effectiveness of a number of electrophoretic methods for the resolution of cheese proteins was compared by Shalabi and Fox (1987) and IDF (1991). In our laboratory, the procedure of Andrews (1983) with a stacking gel is used with very good results; the gels are stained directly with Coomassie G250, as described by Blakesley and Boezi (1977). Figure 2.3 provides an example of the use of such methodology for evaluating interspecies differences in milk protein profiles.

Sodium dodecyl sulphate (SDS)-PAGE, which resolves proteins mainly on the basis of molecular mass, is very effective for most proteins, but since the mass of the four caseins is quite similar, SDS-PAGE is not very effective. β-Casein, which has very high surface hydrophobicity, binds a disproportionately high amount of SDS and, consequently, has a higher electrophoretic mobility than α_{s1}-casein, although it is a larger molecule (Creamer and Richardson, 1984). SDS-PAGE is very effective for the resolution of whey proteins and is the method of choice. The method used in our laboratory is based on that of Laemmli (1970). The more recent advent of advanced proteomic approaches based on traditional gel electrophoresis, such as high-resolution two-dimensional electrophoresis (possibly with mass spectrometry identification/quantification), has proven very effective for protein profiling and for assessing

Fig. 2.3 Urea-PAGE of the milk of various species. Lanes: *1* Macaque monkey; *2* Human milk; *3* African elephant; *4* Rhinocerous; *5* Bovine sodium caseinate; *6* whey protein isolate (Uniacke-Lowe *et al.*, unpublished results)

the pattern and extent of protein hydrolysis in more complex milk protein systems (Mann *et al.*, 2001; Yamada *et al.*, 2002; Manso *et al.*, 2005; Armaforte *et al.*, 2010; Chevalier, 2011b). Figure 2.4 illustrates the use of two-dimensional electrophoresis for detailed analysis of the protein profile of bovine milk.

2.6 Microheterogeneity of the Caseins

It will be apparent from the foregoing discussion that isoelectric casein consists of four principal proteins, α_{s1}-, α_{s2}-, β- and κ-, which represent approximately 38%, 10%, 35% and 15%, respectively, of whole casein. However, SGE or PAGE indicates much greater heterogeneity, which is due to relatively small variations in one of the four principal caseins. These minor variations, referred to as *microheterogeneity*, arise from five factors.

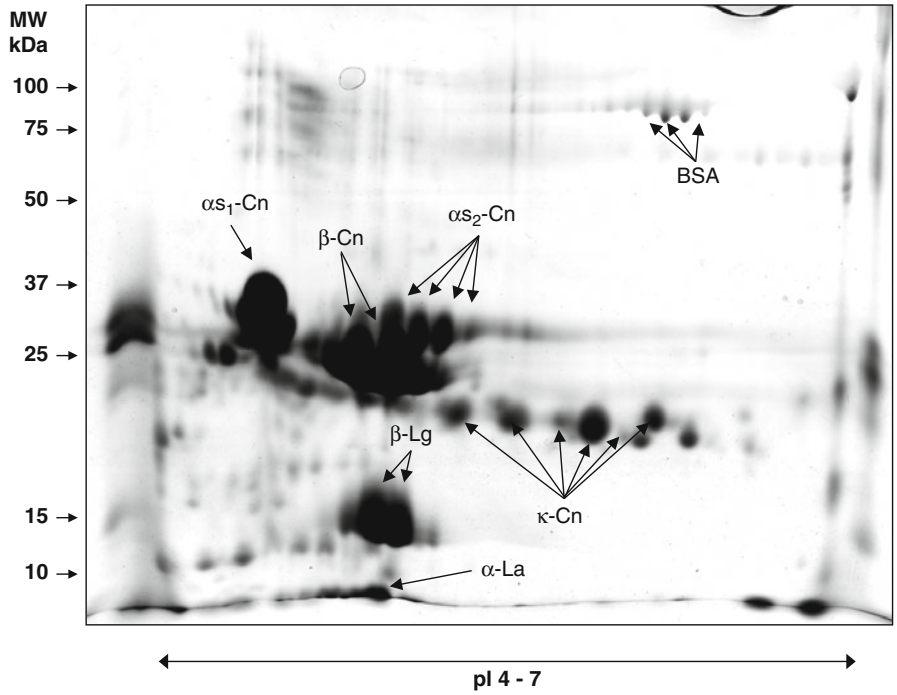


Fig. 2.4 Two-dimensional electrophoretogram of bovine milk under reducing conditions using isoelectric focusing in the range pH 4–7 for the first dimension and a 12% acrylamide gel for the second dimension (Uniacke-Lowe *et al.*, unpublished results)

2.6.1 Variability in the Degree of Phosphorylation

All the caseins are phosphorylated but to a different extent, with each showing variability in the degree of phosphorylation:

Casein	Number of phosphate residues per mole
α_{s1}	8, occasionally 9
α_{s2}	10, 11, 12 or 13
β	5, occasionally 4
κ	1, occasionally 2 or perhaps 3

The number of phosphate residues is indicated thus:

$$\alpha_{s1}\text{-CN 8P, } \beta\text{-CN 5P, etc.}$$

Before the true relationships of the caseins were established, α_{s1}-CN 8P and α_{s1}-CN 9P were referred to as α_{s1} and α_{so}, respectively, and α_{s2}-CN 13P, α_{s2}-CN 12P, α_{s2}-CN 11P and α_{s2}-CN 10P as α_{s2}-, α_{s3}-, α_{s4}- and α_{s6}-, respectively.

2.6.2 Genetic Polymorphism

Aschaffenburg and Drewry (1955) showed that the whey protein, β-lactoglobulin, exists in two forms (variants, polymorphs) A and B, which differ from each other by only two amino acids. The variant found in the milk of any animal is genetically controlled and may be AA, AB or BB, depending on the genetic profile of the parents. The phenomenon referred to as *genetic polymorphism* occurs in all milk proteins, with at least 25 genetic polymorphs of bovine milk proteins known. Since PAGE differentiates on the basis of charge, only polymorphs which differ in charge (i.e. in which a charged residue is replaced by an uncharged one or *vice versa*) are detected; therefore, it is very likely that only a small proportion of the genetic polymorphs of milk proteins have been detected. The genetic polymorph(s) present is indicated by a Latin letter as follows:

$$\beta\text{-CN } \mathbf{A}\text{ 5P}, \alpha_{s1}\text{ - CN } \mathbf{B}\text{ 9P}, \kappa\text{-CN } \mathbf{A}\text{ 1P, etc.}$$

The genetic polymorphism of milk proteins is reviewed in Chap. 15.

2.6.3 Disulfide Bonding

α_{s1}- and β-caseins are devoid of cysteine, but both α_{s2}- and κ-caseins contain two cysteine residues which are involved in intermolecular disulfide bonds. α_{s2}-Casein exists as a disulfide-linked dimer while up to ten κ-casein molecules may be linked by disulfide bonds. As mentioned above, inclusion of a reducing agent in the gel for SGE or PAGE is required for good resolution of κ-casein. In the absence of a reducing agent, α_{s2}-casein appears as a dimer which was originally called α_{s5}-casein.

2.6.4 Variations in the Degree of Glycosylation

κ-Casein is the only member of the casein family which is glycosylated. It contains galactose, *N*-acetylgalactosamine and *N*-acetylneuraminic (sialic) acid, which occur as tri- or tetrasaccharides, the number of which varies from 0 to 4 per molecule of protein (i.e. a total of nine variants).

2.6.5 Hydrolysis of the Primary Caseins by Plasmin

Milk contains several indigenous proteinases, the principal of which is plasmin, a trypsin-like, serine-type proteinase from blood; it is highly specific for peptide bonds with a lysine, or to a lesser extent, arginine, at the N-terminal side of the scissile bond (Kelly and McSweeney, 2003). The preferred casein substrates are β and α_{s2}; α_{s1} is also relatively susceptible, but κ-casein is quite resistant. All the caseins contain several lysine and arginine residues, but only a few bonds are hydrolysed rapidly. The specificity of plasmin on the individual caseins is discussed in Chap. 12. Suffice it to say here that β-casein is hydrolysed rapidly at the bonds Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}. The resulting C-terminal peptides are the γ-caseins (γ^1: β-CN f29–209; γ^2: β-CN f106–209; γ^3: β-CN f108–209), while the N-terminal peptides are included in the proteose peptone fraction (Kelly and McSweeney, 2003).

The γ-caseins represent ~3% of total casein and are readily apparent on PAGE of whole casein (Aimutis and Eigel, 1982). The other plasmin-produced peptides are either too small to be readily detectable by PAGE, or their concentrations are very low relative to the principal caseins. As discussed above under Sect. 2.4, the γ-caseins were among the first components recognized, and it was assumed that they were synthesized as such. Before their true identity was discovered, the three γ-casein components were referred to as follows:

γ^1 - CN : γ-CN;

γ^2 - CN : TS - A and S;

γ^3 - CN : TS - B and R

Note: TS = temperature sensitive, since these peptides are soluble at low temperatures but aggregate on heating; A and B represent genetic variants.

Although α_{s2}-casein in solution is also quite susceptible to plasmin (Le Bars and Gripon, 1989; Visser *et al.*, 1989b), peptides derived from α_{s2}-casein are not evident in milk, probably because they are present at very low concentrations. Although less susceptible to plasmin than α_{s2}- or β-casein, α_{s1}-casein in solution also is hydrolysed readily by plasmin (Le Bars and Gripon, 1993; McSweeney *et al.*, 1993). El-Negoumy (1973) proposed that a minor casein fraction, known as λ-casein, consisting of about nine components which could be resolved by SGE, is produced from α_{s1}-casein by plasmin. These peptides migrate ahead of α_{s1}-casein in alkaline PAGE gels. O'Flaherty (1997) isolated and partially identified seven of these peptides as representing N-terminal fragments of α_{s1}-casein, released by plasmin activity.

2.7 Nomenclature of Milk Proteins

In addition to the genuinely new and unique milk proteins isolated during the period of greatest activity on milk protein research (1950–1970), several other casein (and whey protein) fractions were prepared that were either similar to proteins already isolated and named, were heterogeneous, or were artefacts of the isolation procedure. In order to regularize the nomenclature of the milk proteins, the American Dairy Science Association (ADSA) established a Committee on the Nomenclature, Classification and Methodology of Milk Proteins, which has published seven reports (Jenness *et al.*, 1956; Brunner *et al.*, 1960; Thompson *et al.*, 1965; Rose *et al.*, 1970; Whitney *et al.*, 1976; Eigel *et al.*, 1984; Farrell *et al.*, 2004). The objective of this committee was to develop a flexible nomenclature system that allows for the incorporation of new discoveries arising from the extensive proteomic work conducted to date (and still underway). In addition to simplifying and standardizing the nomenclature of the milk proteins, the characteristics of the various caseins and whey proteins, along with details of the methodologies used to identify and characterize such proteins, are summarized in these articles, which are very valuable references.

The above reports produced by the ADSA Committee on the Nomenclature, Classification and Methodology of Milk Proteins are confined to skim milk proteins, excluding enzymes. Over the last 10–20 years, significant progress has been made in elucidating the primary structures of many of the proteins associated with the fat globule membrane in milk (~1% of the total protein in milk). These scientific developments, along with growing technological and commercial interest in the milk fat globule membrane (MFGM), led to the establishment of a separate review being sponsored by the ADSA Nomenclature Committee on proteins associated with the MFGM (Mather, 2000).

2.8 Whey Proteins

About 20% of the total protein of bovine milk remain soluble at pH 4.6 and are generally referred to as whey (or serum) proteins or non-casein nitrogen; whey contains some phosphopeptides derived from the caseins (i.e. the PPs) which should be classified as derived from the caseins. The whey proteins as a group are readily prepared from milk by any of the methods described for the preparation of casein, i.e. the proteins which are:

- Soluble at pH 4.6
- Soluble in saturated NaCl
- Soluble after rennet-induced coagulation of the caseins
- Separated from the casein micelles by gel filtration or microfiltration
- Are not sedimented by ultracentrifugation, with or without added Ca^{2+}

The composition and properties of products prepared by these various methods differ slightly. Acid whey contains the PP fraction, but no glycomacropeptide produced from κ-casein by rennet action; immunoglobulins are precipitated along with the caseins by saturated NaCl; rennet whey contains the glycomacropeptide from κ-casein, plus small amounts of casein; microfiltration permeates may contain casein monomers (particularly β-casein), in addition to whey proteins, if microfiltration is conducted at <10°C; and small casein micelles remain in the ultracentrifugal serum, especially if Ca is not added. The salt composition of the serum differs very considerably in whey produced by various methods. The whey prepared by any of the above methods, except by gel filtration, contains lactose and soluble salts. For research purposes, purified whey proteins may be prepared from such whey fractions by dialysis or ultrafiltration and freeze-drying the retentates.

On a commercial scale, whey protein-rich products are prepared by:

- Ultrafiltration/diafiltration of liquid whey to remove varying amounts of lactose and other low molecular weight soluble components (e.g., salts and NPN), evaporation and spray-drying to produce WPCs (30–85% protein).
- Ion-exchange chromatography—in which the proteins are adsorbed on an ion exchanger, washed free of lactose and salts, and then eluted with acid or alkali; the protein concentration in the eluates is increased by ultrafiltration, before evaporation and spray-drying to yield WPI, containing ~95% protein.
- Integrated ultrafiltration and microfiltration membrane processing may also be used in the production of WPI. In such processes, ultrafiltration is used, as above, to first concentrate the proteins in liquid whey. During ultrafiltration, any residual fat and phospholipid material from the liquid whey is concentrated along with the protein. Microfiltration is used to remove such fat/phospholipid material from protein concentrates in the production of WPIs.
- Demineralization of whey by electrodialysis or ion-exchange chromatography. In industrial demineralization installations, nanofiltration is often used for pre-concentration and partial demineralization of liquid whey.
- Thermal evaporation of water in the production of whey concentrates.
- Crystallization of lactose, followed by removal of lactose crystals (e.g., using a decanter centrifuge), to concentrate whey proteins in liquid whey.
- Thermal denaturation, removal of precipitated protein, filtration/centrifugation and drying, to yield *lactalbumin*, which has very low solubility and poor functionality.

Several other methods are available for the recovery of whey proteins from whey, but they are not used commercially. Several methods for the purification of the major and minor whey proteins on a commercial scale have also been developed (Mulvihill and Ennis, 2003).

2.9 Fractionation of Whey Proteins

It was recognized early that acid whey contains two well-defined groups of proteins: (1) *lactalbumins*, which are soluble in 50% saturated $(NH_4)_2SO_4$ or saturated $MgSO_4$, and (2) *lactoglobulins*, which are salted-out under these conditions and comprise mainly of immunoglobulins. The lactalbumin fraction was considered homogeneous until Palmer (1934) isolated and crystallized a protein which behaved as an albumin in that it was soluble in half-saturated $(NH_4)_2SO_4$ or saturated $MgSO_4$, but had some characteristics of globulins (i.e. was insoluble in pure water at its isoelectric point (pH 5.2) but was soluble in dilute salt solutions). This protein was identified as the β-peak in free-boundary electrophoretograms of milk proteins; initially it was called *β-lactalbumin* but later renamed *β-lactoglobulin.*

Sorensen and Sorensen (1939) developed several methods for the crystallization of β-lactoglobulin (β-Lg) and also isolated a number of minor (red and green) proteins and a 'crystalline insoluble substance' (CIS) from the mother liquor following crystallization of β-Lg. An improved method for the preparation of CIS from the mother liquor from β-Lg crystallization was developed by Gordon and Semmett (1953). They also showed that the electrophoretic mobility and sedimentation coefficient of CIS were essentially identical to those of the α-peak in electrophoretic and sedimentation patterns of whey and proposed that CIS be called *α-lactalbumin*, although the protein was only slightly soluble in H_2O and, therefore, is not a true albumin.

Polis *et al.* (1950) isolated and crystallized a minor protein from β-Lg mother liquor by fractionation with $(NH_4)_2SO_4$ and ethanol; it is a true albumin and was shown to be identical to *bovine BSA*.

A number of metal-containing proteins, including *lactoperoxidase*, *lactoferrin* and *serum transferrin*, have also been isolated from whey and will be discussed in more detail in Chaps. 10 and 11. Lactoferrin is a major protein in human milk with several biological functionalities.

Several improved procedures for the isolation of α-La and β-Lg have since been developed. Early methods were based on salting-out from whole milk, skim milk, rennet or acid whey, e.g., Aschaffenburg and Drewry (1957) and Armstrong *et al.* (1967). In each procedure, fat and casein, if present, are removed in the first step. Fox *et al.* (1967) exploited the solubility of β-Lg in ~3% TCA while all other proteins are insoluble; this is the easiest of these three methods, and highly purified β-Lg may be prepared by just one step. Since the methods of both Fox *et al.* (1967) and Aschaffenburg and Drewry (1957) may cause denaturation, the method of Armstrong *et al.* (1967) which is performed close to neutrality is recommended but is a rather complicated procedure. Both β-Lg and α-La may be purified by chromatography on Sephadex and/or DEAE-Sephadex or DEAE-cellulose (see McKenzie, 1971b). Genetic variants of β-Lg have been fractionated on DEAE-Sephadex. Methods for the isolation of whey proteins were reviewed by Imafidon *et al.* (1997).

There is considerable interest in the production of most of the major and minor whey proteins on a commercial scale for nutritional or functional applications. Several methods, based on ion-exchange chromatography, membrane filtration technology and thermal, physical and chemical treatments, have been proposed and/or developed for the industrial-scale production of many of the whey proteins (e.g., Amundson *et al.*, 1982; Pearce, 1983; Mailliert and Ribadeau-Dumas, 1988; Stack *et al.*, 1998; Kristiansen *et al.*, 1998; Cheang and Zydney, 2004; Andersson and Mattiasson, 2006; Marella *et al.*, 2011). Many of these approaches used for industrial-scale production of whey protein-enriched ingredients are discussed in more detail by Mulvihill and Ennis (2003).

Individual whey protein-enriched/pure ingredients are commercially available from several dairy ingredient companies. Examples of such ingredients include Bioferrin® (lactoferrin) from Glanbia Nutritionals (Evanston, IL, USA), Hilmar™ 8800 (α-lactalbumin-enriched WPC) from Hilmar Ingredients (Hilmar, CA, USA) and LACPRODAN® OPN-10 (osteopontin) ingredient from Arla Foods Ingredients (Viby, Denmark).

2.10 Some Major Characteristics of Whey Proteins

2.10.1 β-Lactoglobulin

β-Lactoglobulin is a major protein in bovine milk, representing ~50% of whey proteins and 12% of the total protein. It was among the first proteins to be crystallized and, since it could be crystallized readily in large amounts, was for long considered to be homogeneous and a typical globular protein. It has been a favourite subject for protein biochemists and is, therefore, very well characterized. The extensive literature has been reviewed by McKenzie (1971c), Swaisgood (1982), Hambling *et al.* (1992), Sawyer (2003), Kontopidis *et al.* (2004) and Chatterton *et al.* (2006) and is also updated in Chap. 7.

β-Lactoglobulin is the principal whey protein in the milk of the cow, buffalo, sheep and goat, although there are slight interspecies differences (see Chap. 13). At one time, it was considered that β-Lg occurs only in the milk of ruminants, but it is now known that β-Lg occurs in the milk of the sow, mare, kangaroo, dolphin and manatee. However, β-Lg does not occur in human, rat, mouse or guinea pig milk, in which α-La is the principal whey protein.

Four genetic variants, A, B, C and D, of bovine β-Lg have been identified. A fifth variant, which contains carbohydrate, has been identified in the Australian breed, Droughtmaster (Zappacosta *et al.*, 1998). Further variants occur in the milk of yak and Bali cattle (see Chap. 15). Genetic polymorphism also occurs in ovine and caprine β-Lg.

Bovine β-Lg consists of 162 residues per monomer, with a MW of ~18.3 kDa; the amino acid sequence of β-Lg from several species has been established (see Chap. 7). It is rich in sulphur-containing amino acids, which give it a high biological value of 110. It contains two intramolecular disulfide bonds and 1 mol of cysteine per monomer of 18 kDa (Sawyer, 2003). The cysteine is especially important since it reacts, following heat denaturation, with the intermolecular disulfide of κ-casein and significantly affects rennet coagulation and heat stability properties of milk (O'Connell and Fox, 2003). It is also responsible for the cooked flavour of heated milk. Some β-Lgs (e.g., porcine) do not contain a free sulphydryl group. The isoelectric point of bovine β-Lg is ~pH 5.2.

Equine milk contains two isoforms of β-Lg, I and II; like bovine β-Lg, equine β-Lg I contains 162 amino acid residues, but β-Lg II has 163 residues. Equine β-Lg I has a molecular mass of 18.5 kDa and an isoelectric point of pH 4.85, while equine β-Lg II has a molecular mass of 18.3 kDa and an isoelectric point of pH 4.7. In contrast to bovine β-Lg, equine β-Lg contains no free sulphydryl group. Asinine milk also has two forms of β-Lg, β-Lg I and β-Lg II.

β-Lg is a highly structured, compact, globular protein. Optical rotary dispersion and circular dichroism measurements show that, in the pH range 2–6, 10–15% of the molecule exists as α-helices, 43% as β-sheets and 47% as unordered structures, including β-turns. The β-sheets occur in a β-barrel-type structure; each monomer exists almost as a sphere, about 3.6 nm in diameter; its tertiary structure is known (see Chap. 7).

β-Lg shows rather interesting association characteristics (Timasheff and Townend, 1962; McKenzie, 1967; Swaisgood, 1982; Hambling *et al*., 1992; Sawyer, 2003; Kontopidis *et al.*, 2004; de Wit, 2009; see also Chap. 7). Early work indicated that the monomeric MW of β-Lg was ~36 kDa, but it was soon shown that at <pH 3.5 and >pH 7.5, β-Lg dissociates to monomers of 18 kDa. Between pH 5.5 and 7.5, bovine β-Lg forms dimers of MW ~ 36 kDa. Between pH 3.5 and pH 5.2, especially at pH ~4.6, bovine β-Lg A forms octamers of MW ~ 144 kDa. Porcine and other β-Lgs which lack a free thiol do not form dimers; however, lack of a thiol group is probably not directly responsible for the failure to dimerize.

Owing to its high levels of secondary and tertiary structures, β-Lg is very resistant to proteolysis in its native state (Guo *et al.*, 1995), a feature which suggests that the primary function of β-Lg is not nutritional. Indeed, its resistance to proteolysis is the principle behind a method developed for its industrial-scale isolation (Kinekawa and Kitabatake, 1996; Konrad *et al.*, 2000). Since all the other whey proteins have some biological function, it has long been felt that β-Lg might have a biological role. Either or both of two roles have been suggested:

1. It can bind and may act as a carrier for retinol (vitamin A); β-Lg can bind retinol in a hydrophobic pocket, protect it against oxidation and transport it through the stomach to the small intestine where the retinol is transferred to a retinol-binding protein, which has a similar structure to β-Lg. Unanswered questions are how retinol is transferred from the core of the fat globules, where it occurs in milk, to β-Lg and how humans and rodents have evolved without β-Lg. β-Lg is capable of binding many hydrophobic molecules and hence its ability to bind retinol may be incidental. It is a member of the lipocalin family of proteins which contains 14 members, all of which bind hydrophobic molecules (Flower *et al.*, 2000).

2. Through its ability to bind fatty acids, β-Lg stimulates lipase activity, which may be its most important physiological function.

β-Lg is one of the most allergenic proteins in bovine milk for human infants (El-Agamy, 2007), perhaps because human milk lacks β-Lg. β-Lactoglobulin, due to its relative concentration in whey and ease of denaturation on heating, is one of the principal determinants of the physicochemical properties (e.g., thermal gelation) of whey protein ingredients.

2.10.2 Whey Acidic Protein

Whey acidic protein (WAP) was identified first almost 30 years ago in the milk of the mouse and the rat (Hennighausen and Sippel, 1982; Campbell *et al.*, 1984) and has since been found also in the milk of rat, rabbit, camel, wallaby, possum, echidna and platypus. Since the milk of all of these species lacks β-Lg, it was thought that these proteins were mutually exclusive. However, porcine milk, which contains β-Lg, was later found to contain WAP also (Simpson *et al.*, 1998). The MW of WAP is 14–30 kDa (the variation may be due to differences in glycosylation), and it contains two (in eutherians) or three (in monotremes and marsupials) four-disulfide domains (Simpson *et al.*, 2000; Demmer *et al.*, 2001). Since human milk lacks β-Lg, it might be expected to contain WAP, but there are no reports to this effect. In humans and ruminants, the WAP gene has been lost, i.e. a frameshift mutation has transformed the gene into a pseudogene (Rival-Gervier *et al.*, 2003). The physiological function of the WAP protein is still unknown; however, studies of sequence similarity between species (Dandekar *et al.*, 1982) have suggested that it may have a role as a proteinase inhibitor. It has also been hypothesized that WAP is involved in terminal differentiation in the mammary gland (Ikeda *et al.*, 2002) and has antibacterial activity (Tomee *et al.*, 1997; Hagiwara *et al.*, 2003; Yenugu *et al.*, 2004). For more detailed reviews on WAP, see Simpson and Nicholas (2002) and Hajjoubi *et al.* (2006).

2.10.3 α-Lactalbumin

α-Lactalbumin (α-La) represents ~20% of the protein of bovine whey (3.5% of total milk protein) and is the principal protein in human milk (2.2 g L^{-1}). It is a small (MW ~ 14 kDa), well-characterized protein; the literature has been reviewed by Kronman (1989), McKenzie and White (1991), Brew and Grobler (1992), Brew (2003), Chatterton *et al.* (2006) and in Chap. 8.

It contains ~1.9% sulphur, including four intramolecular disulfide bonds per mole. α-La is relatively rich in tryptophan (four residues per mole), thereby giving it a specific absorbance at 280 nm of 20. It contains no cysteine (sulphydryl groups) or phosphate. Its isoionic point is ~pH 4.8, and it has minimum solubility in 0.5 M NaCl at ~pH 4.8 (Brew and Grobler, 1992).

The milk of *Bos taurus* contains only one genetic variant of α-La, B, but Zebu cattle, both in India and Africa, contain two variants, A and B. The B variant contains one arginine residue which is replaced by glutamic acid in α-La A. Both variants have been detected in Droughtmaster cattle.

The primary structure of α-La is homologous with type C lysozyme; of the 123 residues in α-La, 54 are identical to corresponding residues in chicken egg white lysozyme, and 23 more are structurally similar (e.g., serine for threonine and aspartic acid for glutamic acid) (McKenzie and White, 1991). Lysozyme evolved before the divergence of birds and mammals, and α-La appears to be the result of duplication of the lysozyme gene at an early stage of mammalian evolution—it is present in the milk of monotremes. Through its involvement in lactose synthesis (see below), α-La plays a major role in controlling the composition of milk.

α-La is a compact globular protein which exists in solution as a prolate ellipsoid with dimensions of 2.5 nm × 3.7 nm × 3.2 nm. About 26% of the sequence occurs as α-helices, 14% as β-structures and 60% unordered structure (Brew, 2003). It has been difficult to crystallize bovine α-La in a form suitable for X-ray crystallography, which has hampered work on its tertiary structure, but

work on the detailed structure of this protein is at an advanced stage (see Chap. 8).

α-La has been isolated from the milk of the cow, sheep, goat, sow, human, buffalo, rat and guinea pig (see Gordon, 1971; Brew and Grobler, 1992; Brew, 2003; Chap. 8). The milk of some seals contains very little or no α-La. Some minor interspecies differences in the composition and properties have been reported (see Chap. 13).

α-La is a component of lactose synthetase (EC 2.4.1.22), the enzyme which catalyzes the final step in the biosynthesis of lactose:

$$\text{UDP - D - galactose} + \text{D - glucose} \xrightarrow[\text{lactose synthase}]{} \text{lactose} + \text{UDP}$$

Lactose synthetase consists of two dissimilar protein subunits, A and B; the latter protein is α-La, and α-La from many species is effective for bovine lactose synthetase. In the absence of the B protein, the A protein is a non-specific galactosyltransferase, i.e. it transfers the galactose of UDP-galactose to a range of acceptors, but in the presence of α-La, it becomes highly specific (K_M reduced ~1,000-fold) and transfers galactose mainly to glucose to form lactose. α-La is, therefore, a specifier protein, and its action represents a unique form of molecular control in biological reactions. The concentration of lactose in milk is directly related to the concentration of α-La; milk of those seals which lack α-La contains no lactose. Since lactose is responsible for ~50% of the osmotic pressure of milk, its synthesis must be controlled rigidly, and this is possibly the physiological role of α-La. Perhaps, each molecule of α-La regulates lactose synthesis for a short period and is then discarded and replaced. While this is an expensive and wasteful use of an enzyme modifier, the rapid turnover affords a fast response should lactose synthesis need to be altered, as in mastitic infection, when the osmotic pressure increases due to an influx of NaCl from blood.

The activity of α-La in the mammary gland controls the concentration of lactose in milk which in turn determines the movement of water into the milk, and hence the concentration of lactose is inversely related to the concentrations of proteins and lipids in milk (Jenness and Holt, 1987).

α-La is synthesized in the mammary gland, but a very low level is transferred, probably *via* leaky mammocyte junctions, into blood serum, in which the concentration of α-La increases during pregnancy or following administration of steroid hormones to male or female animals (Akers, 2000). The concentration of α-La in blood serum can be used as a reliable, non-invasive indicator of mammary gland development and the potential of an animal for milk production.

Although lactose is the principal carbohydrate in the milk of most species, all milks also contain many oligosaccharides (~130 in human milk), ranging in concentration from trace in bovine milk to 15 g L^{-1} in human milk, and are also present at relatively high concentrations in the milks of monotremes, marsupials and bears (Urashima *et al.*, 2009). The oligosaccharides have lactose (i.e., glucose and galactose) at the reducing end, and many contain fucose and *N*-acetyl neuraminic acid. It is believed that oligosaccharides were produced initially to serve mainly as bactericidal agents for soft-shelled eggs but some was consumed incidentally (Blackburn *et al.*, 1989; see also Chap. 1). Glucose was conserved for other functions, and lactose was not synthesized until the evolution of α-La.

α-La is a metalloprotein; naturally, it binds one Ca^{2+} strongly in a pocket containing four Asp residues; these residues are highly conserved in α-La and in lysozyme, but most c-lysozymes do not bind calcium; an exception is equine milk lysozyme. The Ca-containing bovine α-La protein is quite heat-stable (the most heat-stable of the principal whey proteins), or more correctly, the protein renatures following heat denaturation. (Denaturation does occur at a relatively low temperature, as indicated by differential scanning calorimetry.) When the pH is reduced <5.0, the Asp residues become protonated and lose their ability to bind Ca^{2+}. The apoprotein is denatured and aggregates at quite a low temperature (at ~55°C) and does not renature on cooling. These characteristics of the protein have been exploited in the industrial-scale manufacture of α-La-enriched WPC (Pearce, 1983).

Recently, an interesting non-native state of apo-α-La, stabilized by complex formation with oleic acid, has been found to selectively induce

apoptosis in tumour cells—this complex is known as HAMLET (human α-La made lethal to tumour cells) (Svensson *et al.*, 2000; Pettersson *et al.*, 2006). The complex can be generated from apo--α-La by chromatography on an ion-exchange column, preconditioned with oleic acid. The complex can be formed from human (i.e. HAMLET) or bovine (i.e. BAMLET) apo-α-La (Pettersson *et al.*, 2006), with both forms reported to have comparable cytotoxic activity against three different cancer cell lines (Brinkmann *et al.*, 2011). This complex may offer potential as a premium functional food ingredient.

2.10.4 Blood Serum Albumin

Normal bovine milk (and probably that of all species) contains a low level of BSA (0.1–0.4 $g\,L^{-1}$; 0.3–1.0% of total nitrogen), presumably as a result of leakage from blood. BSA has been studied extensively; reviews include Peters (1985) and Carter and Ho (1994). The MW of the bovine protein is ~66 kDa, and it contains 583 amino acids, the sequence of which is known. The molecule contains 17 disulfides and 1 sulphydryl. All the disulfides link cysteines that are relatively close together in the polypeptide which, therefore, exists as a series of relatively short loops. The molecule is elliptical in shape and is divided into three domains, each containing two longish loops and one short loop. In blood, BSA serves various functions (e.g., ligand binding and free radical trapping), but it has no known function in milk and is probably of little significance although it does bind metals and fatty acids. The latter characteristic may enable it to stimulate lipase activity (see Peters, 1985). The physico-chemical functionality of BSA has been studied extensively as an example of a highly structured but flexible protein (see Mulvihill and Fox, 1989). While BSA has the ability to form heat-induced intermolecular disulfide bonds with α-La and β-Lg (Havea *et al.*, 2000) and influences the denaturation, aggregation and gelation properties of β-Lg (Kehoe *et al.*, 2007), it probably has little effect on the physico-chemical properties of milk protein ingredients due to its relatively low concentration.

2.10.5 Immunoglobulins

Mature bovine milk contains 0.6–1 $g\,L^{-1}$ Ig (~3% of total nitrogen), but colostrum contains up to 10% Igs, the level of which decreases rapidly *postpartum*. Igs are very complex proteins which will not be reviewed here (see texts on Biochemistry or Immunology for reviews and Chap. 9). Essentially, there are five classes of Ig: IgA, IgG (with subclasses, e.g., IgG occurs as IgG_1 and IgG_2), IgD, IgE and IgM. IgA, IgG and IgM are present in milk (Hurley, 2003). IgG consists of two heavy (large) and two light (small) polypeptide chains linked by disulfides (see Chap. 9). IgA consists of two such units (i.e. eight chains) linked by secretory component (SC) and a junction component (J), while IgM consists of five linked four-chain units. The heavy and light chains are specific to each type of Ig.

The physiological function of Ig is to provide immunity to the neonate. Some species, including humans, transfer Igs in utero, and the young are born with a full complement of Igs in its blood (Hurley, 2003). The colostrum of these species contains mainly IgA which is not absorbed by the neonates but functions in the gastrointestinal tract. Ruminants do not transfer Igs in utero, and the neonate is born lacking serum Igs and is very susceptible to infection. Ruminant colostrum contains mainly IgG, which is absorbed in the gastrointestinal tract of the neonate during the first few days *post-partum* and provides passive immunity. Some species, e.g., dog, rat and mouse, transfer Ig both in utero and *via* colostrum (see Chap. 9). Owing to the low concentration of Igs in mature milk, they have little effect on the physico-chemical properties of milk, but the technological and nutritional properties of colostrum and early lactation milk differ substantially from those of mature milk, due partly not only to the presence of Igs but also to an abnormal pH and milk salts. Consequently, such milk is excluded from processing. The modern dairy cow produces much more colostrum than its calf requires with the excess typically fed to older calves and pigs or commercialized (as a liquid or a powder) for feeding orphaned neonates.

Hyperimmunization refers to the immunization of cows with a mixture of non-viable pathogens (i.e. antigens) prior to parturition with the objective of boosting the concentration of immunoglobulins in the milk (particularly colostrum). Such milk is often referred to as 'immune milk' or 'hyperimmune milk'. Interest in this approach dates back to the 1950s, when L.M. Spolverini suggested using bovine colostrum in the diet of infants to confer protection against shared human and bovine diseases (Campbell and Petersen, 1959). Milk powder manufactured from 'immune milk' is commercially available in several markets (e.g., Stolle Milk Biologics Inc., Cincinnati, OH, USA). Claims normally associated with such products include increased resistance to infection, improved immune system and anti-inflammatory properties. However, a study comparing immunoglobulin activity in a colostrum concentrate from non-immunized cows and a milk powder made from milk of hyperimmunized cows showed that both products contained IgG and IgG1 which bound to all the microbial antigens tested but that neither product had anti-inflammatory activity (McConnell *et al.*, 2001). One of the major technological challenges involved in commercializing such products is the conservation of structure and biological activity of the immunoglobulins. Nonthermal processing technologies, such as high pressure (Carroll *et al.*, 2006), have shown promise in this regard.

2.10.6 Proteose Peptones

The proteose-peptone (PP) fraction of milk protein was first recognized by Osborne and Wakeman in 1918 and defined by Rowland (1938) as the 12% TCA-insoluble proteins in acid (pH 4.6) whey prepared from milk heated at 90°C × 30 min. (The principal whey proteins are denatured under these conditions and co-precipitate with the caseins on acidification.) PPs normally represent ~10% of the pH 4.6 soluble nitrogen, but the value increases in late lactation or during mastitis. Initially, the PPs were considered to be indigenous to milk, i.e. not artefacts produced by enzymatic proteolysis or during isolation. The fraction was resolved partially by salting-out methods; the term 'δ-proteose' was introduced to describe the components salted-out by $(NH_4)_2SO_4$. Free-boundary electrophoresis of the PP fraction showed eight peaks, the principal peaks being 3, 5 and 8 which are described as PP3, PP5 and PP8. Gel electrophoresis (SGE or PAGE) of the PP fraction showed that PP8 contains two peptides which were fractionated and named PP8 fast and PP8 slow (PP8f and PP8s). The early literature on the PP is quite confused and was reviewed by McKenzie (1971b) and Parquet (1989).

Characterization of the PP fraction commenced with the work by Brunner and collaborators in the late 1960s and early 1970s (Kolar and Brunner, 1969, 1970; Ng *et al.*, 1970). These authors showed that PP3 is present only in acid whey whereas PP5, PP8s and PP8f partitioned between the casein and whey. PP3 was shown to be a glycoprotein, whereas the other fractions were not. The PP fraction consists of two groups of proteins/peptides:

1. Those derived from caseins by proteolysis, which are now classified with the caseins.
2. A number of minor proteins indigenous to milk, e.g., osteopontin (Sorensen and Petersen, 1993, 1994) and PP3 (Girardet and Linden, 1996), together with trace amounts of lactosylated α-La or β-Lg (Shida *et al.*, 1994).

The principal casein-derived PPs are the N-terminal segments of β-casein produced by the action of plasmin and which complement the three γ-caseins. PP5, PP8s and PP8f are β-casein fragments 1–105/107, 29–105/107 and 1–28, respectively. The PP fraction is much more heterogeneous than was thought originally, and it has been demonstrated to contain as many as 30 peptides. The study on the fractionation and characterization of PPs by O'Flaherty (1997), in addition to confirming the identity of PP5 and PP8f, resolved the peptides β-CN (f29–105) and β-CN (f29–107) and concluded that they do not correspond to PP8s (as claimed by Eigel and Keenan, 1979), but refuted by Andrews and Alichanidis (1983) and Le Roux *et al.* (1995), the identity of which, therefore, remains to be established. O'Flaherty (1997) also isolated and identified

three previously unidentified peptides in the PP fraction: (1) β-CN (f1–38), (2) β-CN (f1–97) and (3) β-CN (f29–97). Formation of the latter two peptides would involve cleavage of the bond Lys_{97}-Val_{98} of β-casein, which had not been shown previously to be a primary plasmin cleavage site although cleavage of all lysine- or arginine-containing bonds in β-casein is possible (Visser *et al.*, 1989a). Formation of β-CN (f1–38) would require the hydrolysis of the bond Gln_{38}-Gln_{39}, which would not be expected to be hydrolysed by plasmin, thereby suggesting that another proteinase may be responsible.

Osteopontin was isolated from the PP fraction of milk protein by Sorensen and Petersen (1993) and characterized by Sorensen and Petersen (1994). This highly phosphorylated, acidic glycoprotein, with a molecular mass of ~60 kDa, has strong calcium-binding properties and is believed to have several important biological activities such as assisting in bone calcification and development of the immune system in infants. Due to these interesting biological activities, strategies have been developed for its enrichment from bovine milk (Sorensen *et al.*, 2001; Sun *et al.*, 2010). Osteopontin is commercially available in a high purity form (LACPRODAN® OPN-10) from Arla Food ingredients (Viby, Denmark).

Unlike the other PPs, PP3 is not derived from casein; the literature on PP3 has been reviewed by Girardet and Linden (1996). The protein was purified by various forms of chromatography, but these failed to yield a homogeneous protein. PAGE showed that most preparations contained two major glycoproteins of MW ~28,000 and 18,000 Da and one or more minor proteins, one of which had a MW of ~11,000 Da. These three proteins were shown to be glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1; 135 amino acid residues; MW ~ 28,000 kDa) and two peptides produced from it by cleavage of the bond Arg_{53}-Ser_{54} by plasmin. PP3 (and GlyCAM-1) appears to be similar to glycoproteins of the MFGM; its carbohydrate moieties are similar to those of butyrophilin, another MFGM protein. Although the amino acid composition of PP3 would indicate that it is not hydrophobic, it does, in fact, behave as a rather hydrophobic protein (as is butyrophilin), possibly owing to the formation of an amphiphilic α-helix, one face of which contains hydrophilic residues, the other face containing hydrophobic ones. It has been referred to as the *hydrophobic fraction of proteose*. PP3 has several interesting functional properties:

- It is heat-stable, aggregates strongly and has very good surface activity. It forms very stable foams and emulsions and is in fact, mainly responsible for the foaming of skim milk. The emulsifying properties of PPs in dairy products such as ice cream and recombined dairy cream have been evaluated recently (Innocente *et al.*, 1998, 2002, 2011; Vanderghem *et al.*, 2007).
- It inhibits spontaneous rancidity, apparently because it reduces interfacial tension between the fat and aqueous phases, and thereby prevents the adsorption of lipase.
- It can insert into cell membranes and play an immunological role.
- It stimulates the growth of bifidobacteria; the best effect is obtained with small peptides (1,000–5,000 Da) and is not due to the carbohydrate moieties.

Recent research has focused on the role of PP fractions of milk as precursors of bioactive proteins and peptides—with the activity of several such fractions having been demonstrated using in vitro studies (Andrews *et al.*, 2006; Mills *et al.*, 2011). Quantification of PPs has been shown to be a promising analytical index in evaluating the ageing of pasteurized and extended shelf-life milks (De Noni *et al.*, 2007).

2.10.7 Nonprotein Nitrogen

The NPN fraction of milk contains those nitrogenous compounds soluble in 12% TCA. It represents ~5% of total nitrogen (~300 mg L^{-1}; 230–420 mg L^{-1}; Harland *et al.*, 1955; Journet *et al.*, 1975). The principal components are summarized in Table 2.2.

The components of the NPN fraction are available nutritionally. Human milk contains a high level of taurine ($H_2NCH_2CH_2$–SO_3H) which can be converted to cysteine and may be nutritionally

Table 2.2 Nonprotein nitrogen of cows' milk (Fox, 2003)

Component	N (mg L^{-1})
Ammonia	6.7
Urea	83.8
Creatinine	4.9
Creatine	39.3
Uric acid	22.8
α-Amino nitrogen	37.4
Unaccounted	88.1

important for infants. Consequently, most modern infant formulae are fortified with taurine. The amino acids in milk support the growth of microorganisms, including LAB used as cultures in the production of cheese and fermented milks. However, the concentration of free amino acids in milk is sufficient to support the growth of LAB to only ~20% of the number required for fermented dairy products. Consequently, LAB depend on a cell envelope-associated proteinase, a complex transport system for peptides and amino acids and a battery of intracellular peptidases to obtain their essential amino acids from casein (Thomas and Pritchard, 1987). Heating to a high temperature (>100°C) leads to the formation of some small peptides from caseins which can support LAB (White and Davies, 1966; Hindle and Wheelock, 1970; Gaucheron *et al.*, 1999).

Urea, the principal constituent of NPN (~50% of NPN), has a very significant effect on the heat stability of milk (Muir and Sweetsur, 1976). The concentration of urea in milk varies considerably, being highest when cows are on fresh pasture, which is reflected in seasonal variations in the heat stability of milk.

2.11 Molecular Properties of the Milk Proteins

The principal milk proteins and many of the minor proteins have been very well characterized at the molecular level and are probably the best characterized of all food protein systems. The principal properties of the six milk-specific proteins are summarized in Table 2.3. A number of features warrant comment.

All six major milk proteins are small molecules, a feature which contributes to their stability. The primary structure of the principal lactoproteins is known and is described in Chaps. 4, 5, 7 and 8. Indeed, the amino acid sequence of the principal proteins, especially β-lactoglobulin and α-lactalbumin, in the milk of several species is known, as are the substitutions in the principal variants (see Chaps. 13 and 15).

The whey proteins are highly structured, but the four caseins lack stable secondary structures; classical physical measurements indicate that the caseins are unstructured, but theoretical considerations indicate that rather than being unstructured, the caseins are very flexible molecules and have been referred to as rheomorphic (Holt and Sawyer, 1993). Current views on the conformation of the caseins are discussed in Chap. 5. The inability of the caseins to form stable structures is due mainly to their high content of the structure-breaking amino acid, proline; β-casein is particularly rich in proline, with 35 of the 209 residues. All the caseins lack intramolecular disulfide bonds, which would reduce the flexibility of the molecules.

The caseins are generally regarded as very hydrophobic proteins, but, as shown in Table 2.3, with the exception of β-casein, they are not exceptionally hydrophobic, rather they have a high surface hydrophobicity, because due to their lack of stable secondary and tertiary structures, most of their hydrophobic residues are exposed.

The open, flexible structure of the caseins renders them very susceptible to proteolysis, which, of course, facilitates their natural function, i.e. as a source of amino acids. Susceptibility to proteolysis is also important in cheese ripening and for the production of protein hydrolysates. In contrast, the whey proteins, especially β-Lg, in the native state are quite resistant to proteolysis, and at least some are excreted in the faeces of infants. This feature is important since most of the whey proteins play a non-nutritional function in the intestine, and, therefore, resistance to proteolysis is important. Most of the milk proteins contain sequences which, when released by proteolysis, are biologically active. Examples of such bioactivity include opioid agonist, ACE inhibitor,

Table 2.3 Chemical composition of the major proteins occurring in the milk of western cattle (Swaisgood, 1982)

Amino Acid	α_{s1}-Casein B	α_{s2}-Casein A	β-Casein A^2	κ-Casein B	γ_1-Casein A^2	γ_2-Casein A^2	γ_3- Casein A	β-Lactoglobulin A	α-Lactalbumin B
Asp	7	4	4	4	4	2	2	11	9
Asn	8	14	5	7	3	1	1	5	12
Thr	5	15	9	14	8	4	4	8	7
Ser	8	6	11	12	10	7	7	7	7
SerP	8	11	5	1	1	0	0	0	0
Glu	24	25	18	12	11	4	4	16	8
Gln	15	15	21	14	21	11	11	9	5
Pro	17	10	35	20	34	21	21	8	2
Gly	9	2	5	2	4	2	2	3	6
Ala	9	8	5	15	5	2	2	14	3
1/2 Cys	0	2	0	2	0	0	0	5	8
Val	11	14	19	11	17	10	10	10	6
Met	5	4	6	2	6	4	4	4	1
His	11	11	10	13	7	3	3	10	8
Leu	17	13	22	8	19	14	14	22	13
Tyr	10	12	4	9	4	3	3	4	4
Phe	8	6	9	4	9	5	5	4	4
Trp	2	2	1	1	1	1	1	2	4
Lys	14	24	11	9	10	4	3	15	12
His	5	3	5	3	5	4	3	2	3
Arg	6	6	4	5	2	2	2	3	1
PyroGlu	0	0	0	1	0	0	0	0	0
Total residues	199	207	209	169	181	104	102	162	123
Molecular weight	23,612	25,228	23,980	19,005	20,520	11,822	11,557	18,362	14,174
$H\Phi_{ave}$	(kJ/residue)	4.89	5.12	4.64	5.58	5.85	6.23	6.29	5.03

immunomodulator, mineral binding and antimicrobial (FitzGerald and Meisel, 2003; Phelan *et al.*, 2009; Mills *et al.*, 2011).

Owing to their high hydrophobicity, the milk proteins, especially the caseins, have a propensity to yield bitter hydrolysates which is problematic in the production of dietetic products and cheese, in which bitterness may be a problem unless precautions are taken. One of the most notable features of the amino acid sequence of the caseins is that the hydrophobic and hydrophilic residues are not distributed uniformly, thereby giving the caseins a distinctly amphiphatic structure. This feature, coupled with their open flexible structure and hydrophobicity, gives the caseins good surface activity and good foaming and emulsifying properties, making casein the functional protein of choice for many applications.

Also owing to their open structure, the caseins have a high specific volume and, consequently, form highly viscous solutions, which is a disadvantage in the production of caseinates. The viscosity of sodium caseinate solutions is so high that it is not possible to spray-dry solutions containing >20% protein, thereby increasing the cost of drying and resulting in low-bulk density powders. However, high viscosity is desirable in certain applications, e.g., emulsion stabilization.

The lack of stable tertiary structures means that the caseins are not denaturable *sensu stricto* and, consequently, are extremely heat-stable; sodium caseinate, at pH 7, can withstand heating at 140°C for several hours without visible change, while unconcentrated milk is stable at 140°C, pH 6.7 for 20 min (Fox, 1981). This very high heat stability makes it possible to produce heat-sterilized dairy products with very little change in physical appearance; no other major food system would withstand such severe heating without undergoing major physical and sensoric changes.

The caseins have a very strong tendency to associate; even in sodium caseinate, the most soluble form of casein, the molecules are present as aggregates of 250–500 kDa, i.e. containing 10–20 molecules (Pepper, 1972; Pepper and Farrell, 1982). Association is due mainly to hydrophobic bonding. One of the undesirable consequences of this strong association is the difficulty in isolating and analyzing the caseins, for which a dissociating agent, e.g., urea or SDS, is required. On the other hand, a tendency to associate is important for some functional applications and in the formation and stabilization of casein micelles (see Sect. 2.12). In contrast, the whey proteins are molecularly dispersed in solution.

Owing to their high content of phosphate groups, which occur in clusters, α_{s1}-, α_{s2}- and β-caseins have a strong tendency to bind metal ions, which in the case of milk are mainly Ca^{2+}. This property has many major consequences; the most important from a technological viewpoint is that these three proteins, which represent ~85% of total casein, are insoluble at Ca^{2+} concentrations >~6 mM at temperatures >20°C. Since bovine milk contains ~30 mM Ca^{2+}, one would expect that the caseins would precipitate under the conditions prevailing in milk. However, κ-casein, which contains only one organic phosphate group, binds Ca^{2+} weakly and is soluble at all Ca concentrations found in dairy products. Furthermore, when mixed with the Ca-sensitive caseins, κ-casein can stabilize and protect ~10 times its mass of the former by forming large colloidal particles referred to as casein micelles, which are discussed in Sect. 2.12. The micelles act as carriers of inorganic elements, especially Ca and P, but also Mg and Zn, and are, therefore, very important from a nutritional viewpoint. Through the formation of micelles, it is possible to solubilise much higher levels of Ca and PO_4 than would otherwise be possible. Without casein to stabilize CCP, much of this salt present in bovine and other milks would precipitate in the ducts of the mammary gland, causing blockages which may result in the death of mammary cells, the whole organ or even the animal.

The three calcium-sensitive caseins are distinctly different proteins with a very low level of homology (Fig. 2.5). Why milk contains three calcium-sensitive caseins is not obvious—they are presumably not the result of gene duplication. The evolution of multiple calcium-sensitive caseins is quite ancient—monotreme milk contains α_s- and β-caseins, but the milk of at least some marsupials (e.g., tamer wallaby) lacks α_s-casein as do human milk, the milk of other

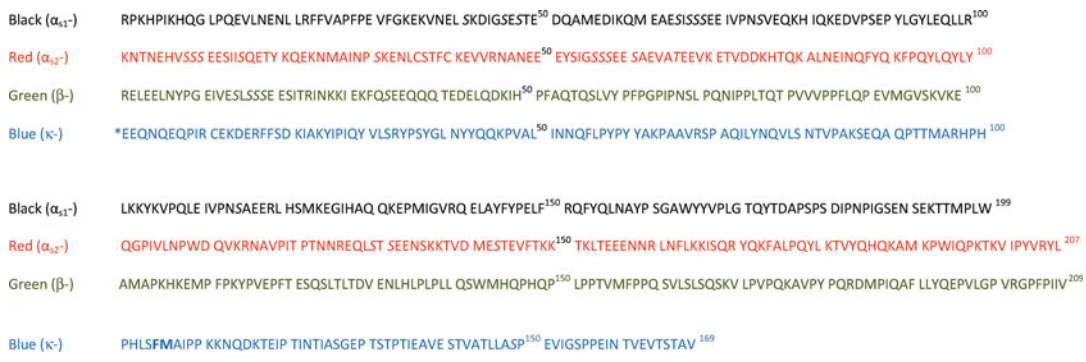


Fig. 2.5 Amino acid sequences of bovine α_{s1}-, α_{s2}-, β- and κ-casein (Swaisgood, 2003)

primates and that of some goats and sheep. Only cattle and buffalo produce two distinctly different α_s-proteins, although many secrete α_s-caseins varying in the level of phosphorylation (e.g., horse and donkey).

2.12 Casein Micelle

About 95% of the casein of milk exists as large aggregate colloids known as micelles. The dry matter of the micelles is ~94% protein and 6% low molecular mass species, referred to collectively as CCP, and consisting mainly of calcium and phosphate with small amounts of magnesium and citrate and trace amounts of other species. The micelles are highly hydrated, binding ~2.0–4.0 g H_2O g^{-1} protein (depending on how hydration is measured). It has been known since the late nineteenth century that the caseins exist as large colloidal particles which are retained by Pasteur-Chamberland porcelain filters (roughly equivalent to modern ceramic microfiltration membranes) (see Kastle and Roberts, 1909). These particles scatter light and are mainly responsible for the white colour of milk (the small fat globules also scatter light weakly); they can be 'visualized' by the ultramicroscope (essentially a device for measuring light scattering). The milk of all species is white, suggesting that all contain casein micelles. The white colour is lost if the micelle structure is disrupted, e.g., by dissolving CCP by addition of citrate, EDTA or oxalate, by increasing pH or by adding urea (>5 M) or ethanol (~35% at 70°C).

Table 2.4 Average characteristics of casein micelles (Fox, 2003)

Characteristic	Value
Diameter	130–160 nm
Surface	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g/cm^3
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O/g protein
Voluminosity	4.4 cm^3/g
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^4 Da
Number of peptide chains (MW: 30,000 Da)	10^4
Number of particles per mL milk	10^{14}–10^{16}
Whole surface of particle	5×10^4 cm^2/mL milk
Mean free distance	240 nm

Some of the principal properties are summarized in Table 2.4.

Electron microscopy shows that casein micelles are generally spherical in shape. The diameter of bovine casein micelles ranges from 50 to 500 nm (average ~120 nm), and they have a mass ranging from 10^6 to 3×10^9 Da (average ~10^8 Da). There are very many small micelles, but these represent only a small proportion of the mass. The micelles in human milk are quite small (~60 nm in diameter) while those in equine or asinine milk are very large (~500 nm), i.e. the micelles in equine milk are 70 times larger than bovine casein micelles. Bovine milk contains 10^{14}–10^{16} micelles mL^{-1} milk, and they are roughly two micelle diameters apart, i.e. they are

quite tightly packed. Since the milk of lagomorphs contains ~20% protein, the micelles must be very closely packed (the size of the micelles in lagomorph milk is unknown).

2.12.1 Stability of Casein Micelles

The micelles are quite stable to the principal processes to which milk is normally subjected:

- They are very stable at high temperatures, coagulating only at 140°C × 15–20 min at the normal pH of milk. Such coagulation is not due to protein denaturation *sensu stricto* but to changes which cause a decrease in pH due to the pyrolysis of lactose to various acids, dephosphorylation of the casein, cleavage of the carbohydrate-rich moiety of κ-casein, denaturation of the whey proteins and their precipitation on the casein micelles and precipitation of soluble calcium phosphate on the micelles at the higher temperatures.
- They are stable to compaction (e.g., they can be sedimented by ultracentrifugation and redispersed readily by mild agitation).
- They are stable to conventional, commercial homogenization. However, casein micelles are partially disrupted by high-pressure homogenization, as evidenced by decreases in casein micelle size on single-stage high-pressure homogenization at 41–350 MPa (Sandra and Dalgleish, 2005; Roach and Harte, 2008).
- Casein micelles are unstable to high-pressure processing, particularly at pressures in excess of 200 MPa. Several studies have shown changes in casein micelle size (by up to ~50%) on treatment of raw and reconstituted milk at 250–600 MPa (Desobry-Banon *et al.*, 1994; Gaucheron *et al.*, 1997; Needs *et al.*, 2000). Casein micelle instability resulting in decreases in casein micelle size is thought to be largely due to high-pressure-induced partial dissolution of CCP (Huppertz *et al.*, 2002), while instability resulting in increased micelle size is thought to be due to the formation of casein aggregates (Huppertz *et al.*, 2004). The changes in micelle size and stability which occur during high-pressure treatment are temperature-dependent (Gaucheron *et al.*, 1997), due to the effect of temperature on pressure-induced whey protein-casein interactions. α-Lactalbumin and β-lactoglobulin are denatured by high-pressure treatment, with levels of denaturation of β-lactoglobulin reaching 70–80% after treatment of milk at 400 MPa (Scollard *et al.*, 2000).
- On cooling of skim milk to temperatures in the range 0–5°C, a limited (up to ~20%) proportion of total β-casein (and indeed other caseins) dissociates from the micelles (Rose, 1968; Downey and Murphy, 1970; Creamer *et al.*, 1977). The effect of lowering temperature on the solubilization of β-casein is presumably due to weakening of the strength of hydrophobic interactions between β-casein molecules or other caseins, which may act as integral components of the casein micelle structure (Swaisgood, 2003).
- Slow freezing and storage of milk at temperatures in the range −10°C to −20°C can cause some destabilization (cryodestabilization) due to an increase in Ca^{2+} concentration in the unfrozen phase of milk and a decrease in pH, due to precipitation of $Ca_3(PO_4)_2$. Cryodestabilized casein can be dispersed in water to give particles with micelle-like properties, which have not been fully characterized (Moon *et al.*, 1988).
- Concentration of milk by ultrafiltration, evaporation and spray-drying can cause destabilization of casein micelles, with the extent of destabilization generally increasing with increasing concentration factor. The close packing of casein micelles, increases in Ca^{2+} concentration and decreases in pH, caused by precipitation of $Ca(H_2PO_4)_2$ and $CaHPO_4$ as $Ca_3(PO_4)_2$ (releasing H^+), are the main factors responsible for destabilization of casein micelles on concentration (Oldfield *et al.*, 2005; Havea, 2006; Karlsson *et al.*, 2007; Martin *et al.*, 2007; Fox and Brodkorb, 2008).
- Casein micelles are stable to high Ca^{2+} concentration, at least up to 200 mM at temperatures up to 50°C.
- The caseins aggregate and precipitate from solution when the pH is reduced to the isoelectric point of casein (pH 4.6). Precipitation at this pH is temperature-dependent (i.e. does

not occur at temperatures <5–8°C) and occurs over a wide pH range, perhaps 3.0–5.5 at higher temperatures; micelles probably do not exist <pH 5 owing to the solution CCP and perhaps other factors.

- As the pH of milk is reduced, CCP dissolves and is fully dissolved ≤pH 4.9; acidification of cold (4°C) milk to pH 4.6, followed by dialysis against bulk milk, is a convenient and widely used technique for changing the CCP content of milk (Pyne and McGann, 1960). If undialyzed, acidified cold milk is readjusted to pH 6.7, the micelles reform provided that the pH had not been reduced below 5.2. This property seems to suggest that most of the CCP can be dissolved (removed) without destroying the structure of the micelles.
- Some proteinases catalyze a very specific hydrolysis of κ-casein, as a result of which the casein coagulates or gels in the presence of Ca^{2+} or other divalent ions (Lucey, 2011). This is the key step in the manufacture of most cheese varieties.
- At room temperature, the micelles are destabilized by ~40% ethanol at pH 6.7 and by lower concentrations if the pH is reduced (Horne, 2003). However, if the system is heated to ~70°C, the precipitate redissolves, and the system becomes translucent. When the system is recooled, the white appearance of milk is restored, and a gel is formed if the ethanol-milk mixture is held at 4°C, especially if a concentrated (>2×) milk was used. If the ethanol is removed by evaporation, very large aggregates (average diameter ~3,000 nm) are formed which have very different properties from those of natural micelles. The aggregates can be dispersed to particles of average diameter ~500 nm. The dissociating effect of ethanol is promoted by increasing the pH (35% ethanol causes dissociation at 20°C at pH 7.3) or adding NaCl. Methanol and acetone have a dissociating effect similar to ethanol, but propanol causes dissociation at ~25°C. The mechanism by which ethanol and similar compounds cause the dissociation of casein micelles has not been established, but it is not due to the solution of CCP, which is unchanged.
- The micelles are also dissociated by urea (5 M), SDS or raising the pH to >9 (McGann and Fox, 1974; Lefebvre-Cases *et al.*, 1998; De Kruif and Holt, 2003). Under these conditions, the CCP is not dissolved; in fact, increasing the pH increases the level of CCP. If the urea is removed by dialysis against a large excess of bulk milk, micelles are reformed, but these have not been characterized adequately (McGann and Fox, 1974).

2.12.2 Micelle Structure

The structure of the casein micelles has attracted the attention of scientists for many years. Knowledge of micelle structure is important because reactions undergone by the micelles are central to many dairy processing operations (e.g., cheese manufacture; stability of sterilized, sweetened-condensed and reconstituted milks and frozen products). From the academic viewpoint, the casein micelle presents an interesting and complex problem in protein quaternary structure.

It was recognized early that the caseins in milk exist as large colloidal particles, and there was some speculation on the structure of these particles and how they were stabilized (Alexander, 1910; Linderstørm-Lang and Kodama, 1929; Eilers *et al.*, 1947; McMeekin and Polis, 1949; Lindqvist, 1963). No significant progress was possible until the isolation and characterization of κ-casein (Waugh and von Hipple, 1956). The first attempt to describe the structure of the casein micelle was that of Waugh (1958), and since then, a considerable amount of research effort has been devoted to elucidating the structure of the casein micelle. This work is summarized here.

The principal features which must be met by any micelle model are:

- κ-Casein, which represents ~15% of total casein, must be located so as to be able to stabilize the calcium-sensitive α_{s1}-, α_{s2}-, and β-caseins, which represent ~85% of total casein.
- Chymosin and similar proteases, which are relatively large molecules (~35 kDa), very rapidly and specifically hydrolyse most of the κ-casein.

- When heated in the presence of whey proteins, as in normal milk, κ-casein and β-lactoglobulin (MW = 36 kDa in milk) interact to form a complex which modifies the properties of the micelles, e.g., rennet and heat coagulation.

The arrangement that would best meet these requirements is a surface layer of κ-casein surrounding the Ca-sensitive caseins, somewhat analogous to a lipid emulsion in which the triglycerides are surrounded by a thin layer of emulsifier. Removal of CCP results in disintegration of the micelles into particles of MW ~ 10^6 Da, suggesting that CCP is a major integrating factor in the micelles. The properties of the CCP-free system are very different from those of normal milk (e.g., it is sensitive to and precipitated by relatively low levels of Ca^{2+}, it is more stable to heat-induced coagulation and it is not coagulable by rennets). Many of these properties can be restored, at least partially, by increased concentrations of calcium. However, CCP is not the only integrating factor, as indicated by the dissociating effect of temperature, urea, SDS, ethanol or alkaline pH. As the temperature is lowered, casein, especially β-casein, dissociates from the micelles (Rose, 1968); the amount of β-casein which dissociates varies from 10 to 50% depending on the method of measurement; it increases to a maximum at ~pH 5.2.

Various models of casein micelle structure have been proposed over the last 50 years. They have been refined progressively as more information has become available. Progress has been reviewed regularly, e.g., Rose (1969), Garnier and Ribadeau-Dumas (1970), Waugh (1971), Garnier (1973), Farrell (1973), Slattery and Evard (1973), Farrell and Thompson (1974), Slattery (1976), Schmidt (1980, 1982), Payens (1979, 1982), Walstra and Jenness (1984), McMahon and Brown (1984), Ruettimann and Ladisch (1987), Rollema (1992), Visser (1992), Holt (1992, 1994), Walstra (1990, 1999), Holt and Horne (1996), Horne (1998, 2002, 2006, 2011), McMahon and McManus (1998), de Kruif (1999), de Kruif and Holt (2003), McMahon and Oommen (2008) and Dalgleish (2011).

The models fall into three general categories:

1. Core-coat
2. Internal structure
3. Sub-micelles; in many of the models in this category, it is proposed that the sub-micelles have a core-coat structure

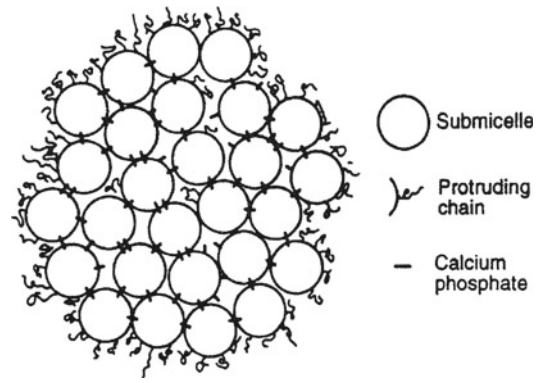


Fig. 2.6 Sub-micelle model of the casein micelle (from Walstra and Jenness, 1984)

Many of the earlier models proposed that the micelle is composed of sub-micelles of MW ~ 10^6 Da and 10–15 nm in diameter (Fig. 2.6). This type of model was first proposed by Morr (1967). The sub-micelles are believed to be linked together by CCP, thereby giving the micelle an open, porous structure. On removal of CCP, (e.g., by acidification/dialysis, EDTA, citrate or oxalate), the micelles disintegrate. Disintegration may also be achieved by treatment with urea, SDS, 35% ethanol at 70°C or pH > 9. These reagents do not solubilise CCP, suggesting that other forces (e.g., hydrophobic and hydrogen bonds) contribute to micelle structure. The particles (sub-micelles) produced by these various agents have not been compared, and the effect of combinations of these agents has not been reported; since they function by different mechanisms, their effects should be cooperative.

The structure of the sub-micelle remains a contentious issue. Waugh *et al.* (1970) proposed a rosette-type structure very similar to that of a classical soap micelle. It was proposed that the polar regions of α_s- (α_{s1}-, α_{s2}-) and β-caseins are orientated toward the outside of the sub-micelle to reduce electrostatic repulsion between neighbouring charged groups and that each sub-micelle is surrounded by a layer (coat) of κ-casein which also provides a κ-casein coat for the entire micelle. The role of CCP was not considered in the development

of this model, which was a major weakness. Also, it is difficult to explain by this model how part of the β-casein dissociates on cooling.

Payens (1966) proposed a model in which β-casein associates to form long thread-like structures to which α_s- (α_{s1}- and α_{s2}-) casein is associated hydrophobically to form the core of the micelle which is surrounded by a layer of κ-casein and CCP.

A variation of this model was proposed by Rose (1969), who suggested that threads of polymerized β-casein form the matrix of the sub-micelles to which α_s- (α_{s1}- and α_{s2}) caseins are attached by hydrophobic bonding; each sub-micelle was considered to be surrounded by a layer of κ-casein, some of which is buried within the micelle where it is inaccessible to chymosin. The structure of each sub-micelle was considered to be stabilized by CCP, which also cements neighbouring sub-micelles to form an intact micelle. The dissociation of casein, especially β-casein, and the important role of κ-casein in micelle structure and function can be explained readily by this model.

Slattery and Evard (1973) and Slattery (1976) proposed that the sub-micelles are not covered completely by a layer of κ-casein and that there are κ-casein-rich, hydrophilic regions on the surface of each sub-micelle. The latter aggregate *via* the hydrophobic patches such that the entire micelle assumes a κ-casein-rich surface layer; but some of the other caseins are also on the surface.

This model was elaborated further by Schmidt (1980, 1982), who suggested that the κ-casein content of sub-micelles varies and that the κ-casein-deficient sub-micelles are located in the interior of the micelle, with the κ-casein-rich sub-micelles concentrated on the surface, thereby giving the overall micelle a κ-casein-rich surface layer. This model was refined further by Walstra and Jenness (1984) and Walstra (1990, 1999), who proposed that the hydrophilic C-terminal region of κ-casein protrudes from the surface, forming a layer 5–10 nm thick and giving the micelles a hairy appearance. This hairy layer is responsible for micelle stability through major contributions to zeta potential (−20 mV) and steric stabilization. The idea of a steric stabilizing layer of κ-casein traces back to Hill and Wake (1969), who considered the amphiphilic structure of κ-casein to be an important feature of its micelle-stabilizing properties. If the hairy layer is removed (e.g., through specific hydrolysis of κ-casein) or collapsed (e.g., by ethanol), the colloidal stability of the micelles is destroyed, and they coagulate or precipitate (see Holt and Horne, 1996).

A further variation of the sub-unit model is that of Ono and Obata (1989), who proposed two types of subunits—one consisting of α_s- (α_{s1}- and α_{s2}-) and β-caseins, which are present in the core, and some of α_s- (α_{s1}- and α_{s2}-) and κ-caseins, which form a surface layer. An attempt to elucidate the internal structure of the sub-micelles was made by Kimura *et al.* (1979), who proposed that the casein polypeptides were folded within the sub-micelles such that the hydrophilic portions are at the surface with the hydrophobic sections in the interior but without preferential distribution of the casein types.

Although the sub-micelle model of the casein micelle adequately explains many of the principal features of, and physico-chemical reactions undergone by, the micelles and has been supported widely, it has never enjoyed unanimous support and alternative models have been proposed. Visser (1992) proposed that the micelles are spherical conglomerates of casein molecules randomly aggregated and held together partly by salt bridges in the form of amorphous calcium phosphate and partly by other forces (e.g., hydrophobic bonds) with a surface layer of κ-casein. Holt (1992, 1994) depicted the casein micelle as a tangled web of flexible casein molecules forming a gel-like structure in which micro-granules of CCP are an integral feature and from the surface of which the C-terminal region of κ-casein extends, forming a hairy layer (Fig. 2.7). These two models retain two of the key features of the sub-micellar model (i.e. the cementing role of CCP and the predominantly surface location and micelle-stabilizing role of κ-casein) and differ from it mainly with respect to the internal structure of the micelle. Dalgleish (1998) agreed that the micellar surface is only partially covered with κ-casein, which is distributed non-uniformly on the surface. This surface coverage provides steric stabilization

against the approach of large particles, such as other micelles, but the small-scale heterogeneities and the gaps between κ-casein molecules provide relatively easy access for molecules with dimensions of individual proteins or smaller.

Much of the evidence for a sub-micellar structure came from electron microscopy studies, such as that of Knoop *et al.* (1979), which appeared to show variations in electron density, which was interpreted as indicating sub-micelles, i.e. a raspberry-like structure. However, artefacts may arise in electron microscopy owing to fixation, exchanging water for ethanol, air drying or metal coating. Using a new cryopreparation electron microscopy stereo-imaging technique, McMahon and McManus (1998) found no evidence to support the sub-micellar model and concluded that if the micelles do consist of sub-micelles, these must be smaller than 2 nm or less densely packed than previously presumed. The TEM micrographs appear very similar to the model prepared by Holt (1994). Holt (1998) concluded that none of the sub-micelle models of casein micelle structure explained the results of gel permeation chromatography of micelles dissociated by removal of CCP or by urea. de Kruif (1998) supports the structure of the casein micelle as depicted by Holt (1992, 1994) and describes the behaviour and properties of the micelles in terms of adhesive hard spheres.

Fig. 2.7 Model of the casein micelle (modified from Holt, 1994)

At the other extreme of proposed models of the casein micelle is that of Parry and Carroll (1969) who suggested that κ-casein forms the core (nucleus) of the micelle, surrounded by α_s- (α_{s1}- and α_{s2}-) and β-caseins. The model of Garnier and Ribadeau-Dumas (1970) might be regarded as a variant of this: κ-casein was considered to form nodes in a three-dimensional network in which the branches were proposed to consist of copolymers of α_s- (α_{s1}- and α_{s2}-) and β-caseins.

A more recent model for casein micelle structure is the 'dual-binding' model put forward by Horne (1998). This model suggests that micelle structure is governed by a balance of hydrophobic interactions and CCP-mediated cross-linking of hydrophilic regions (Fig. 2.8).

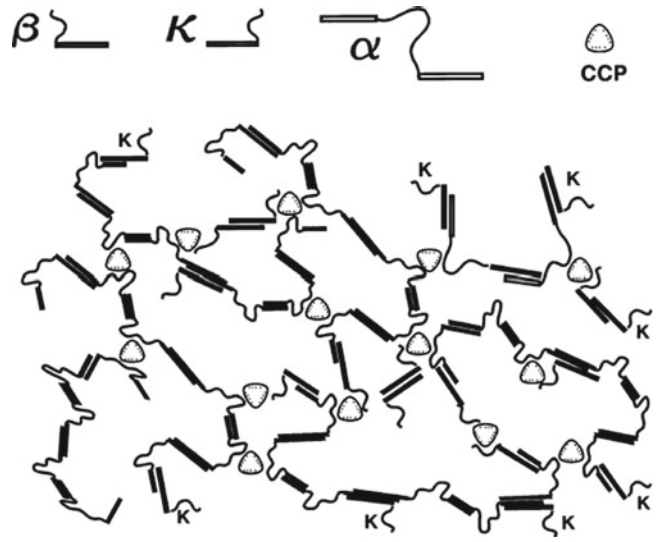


Fig. 2.8 Dual-bonding model of the casein micelle (from Horne, 1998)

The study of casein micelle structure continues to be an active and exciting area of research with developments in analytical approaches contributing new information about casein micelle structure and stability (see Bouchoux *et al.*, 2010).

More detailed information on the structure of the casein micelle is presented in Chap. 6.

2.13 Interspecies Comparison of Milk Proteins

This chapter has been concerned mainly the protein system of bovine milk, which is by far the most important commercially. However, there are ~4,500 species of mammal, each of which produces milk, the composition and properties of which are more or less species-specific. Unfortunately, the milk of most species has not been studied at all; some information is available on the milk of ~180 species. However, the data on the milk of only about 50 species are considered to be reliable, in that a sufficient number of samples was analyzed and that these samples were reliable, properly taken and covering the lactation period adequately. Milk from the commercially important species, cow, goat, sheep, buffalo, yak, horse and pig, is quite well characterized. For medical and nutritional reasons, human milk is also well characterized, as is that of experimental laboratory animals, especially rats and mice. General reviews on non-bovine milks include Macy *et al.* (1950), Evans (1959), Laxminarayana and Dastur (1968), Jenness and Sloan (1970), Rao *et al.* (1970), Woodward (1976), Jenness (1973, 1979, 1982), Addeo *et al.* (1977), Farah (1993), Solaroli *et al.* (1993), Atkinson and Lonnerdal (1989), Jensen (1995), Rudloff and Kunz (1997), Kappler *et al.* (1998), Verstegen *et al.* (1998), Martin *et al.* (2003), Park *et al.* (2007), Raynal-Ljutovac *et al.* (2007), Silanikove *et al.* (2010), Uniacke-Lowe *et al.* (2010) and Uniacke and Fox (2011).

The milk of the species for which data are available shows considerable differences in protein content, i.e. from ~1 to 20%. The protein content reflects the growth rate of the neonate of the species, i.e. its requirements for essential amino acids. The milk of all species for which data are available contains two groups of protein, caseins and whey proteins. Both groups show genus- and even species-specific characteristics which presumably reflect some unique nutritional or physiological requirements of the neonate of the species. Interestingly, and perhaps significantly, of the milks that have been characterized, human and bovine milks are more or less at opposite ends of the spectrum.

There is considerably more and better information available on the interspecies comparison of individual milk proteins than of overall milk composition; this is not surprising since only one sample of milk from one animal is sufficient to yield a particular protein for characterization in addition to advances in DNA homology studies. The two principal milk-specific whey proteins, α-La and β-Lg, from quite a wide range of species have been characterized, and, in general, show a high degree of homology (see Chaps. 7 and 8). However, the caseins show much greater interspecies diversity, especially in the α-casein fraction—all species that have been studied appear to contain a protein that has an electrophoretic mobility similar to that of bovine β-casein (O'Connor and Fox, 1970), but the β-caseins that have been sequenced show a low level of homology (Holt and Sawyer, 1993). Human β-casein occurs in multi-phosphorylated form (0–5 mol P per mol protein; see Atkinson and Lonnerdal, 1989), as does equine β-casein (Ochirkuyag *et al.*, 2000). Considering the critical role played by κ-casein, it would be expected that all casein systems contain this protein. Human κ-casein is very highly glycosylated, containing 40–60% carbohydrate (compared with approximately 10% for bovine κ-casein), which occurs as oligosaccharides which are much more diverse and complex than those in bovine milk (see Atkinson and Lonnerdal, 1989).

The α_s-casein fraction differs markedly between species (O'Connor and Fox, 1970; Martin *et al.*, 2003); human milk probably lacks an α_s-casein while the α-casein fractions in horse and donkey milk are very heterogeneous. The caseins of only about ten species have been studied in some detail. In addition to the references cited earlier in this section, the literature has been reviewed by Ginger and Grigor (1999), Martin

et al. (2003) and in Chap. 13. The bibliography in Chap. 13 includes numerous references on the proteins and milk protein genes from several species. There are very considerable interspecies differences in the minor proteins of milk. The milks of those species which have been studied in sufficient depth contain approximately the same profile of minor proteins, but there are very marked quantitative differences. Most of the minor proteins in milk have some biochemical or physiological function, and the quantitative interspecies differences presumably reflect the requirements of the neonate of the species. Many of the minor milk proteins are considered in Chaps. 9, 10 and 11. Where information is available, interspecies comparisons are made in these chapters.

In the milk of all species, the caseins probably exist as micelles (at least the milks appear white), but the properties of the micelles in the milk of only a few species have been studied. The micelles in caprine milks were studied by Ono and Creamer (1986). The water buffalo is the second most important dairy animal and is particularly important in India. The composition and many of the physico-chemical properties of buffalo milk differ considerably from those of bovine milk (see Patel and Mistry, 1997, for references). Other properties of buffalo milk will be mentioned for comparative purposes in other chapters. Some properties of the casein micelles in camel milk have been described by Attia *et al.* (2000). Possibly because porcine milk is relatively easily obtained, but also because it has interesting properties, the physico-chemical behaviour of porcine milk has been studied fairly thoroughly and the literature reviewed by Gallagher *et al*. (1997). Equine and asinine milks have also been the subject of some detailed characterization over the last 20 years or so (Oftedal and Jenness, 1988; Salimei *et al.*, 2004; Uniacke-Lowe *et al.*, 2010; Uniacke and Fox, 2011).

2.14 Summary and Perspective

Research on milk proteins commenced about 200 years ago, before the word 'protein' was coined. Progress was slow during the first 100 years, during which it was recognized that there are three groups of proteins in milk: caseins, albumins and globulins, the first being milk-specific but it was thought that the latter two were derived from blood. It was also realized that casein exists in milk as large calcium- and phosphate-rich aggregates/particles, now known as casein micelles, a term which was introduced in 1920. Facilitated by the introduction of new analytical techniques in protein chemistry, there has been a succession of developments and refinements, of which the following are probably the most important.

Although research in the 1920s suggested that acid casein was heterogeneous, the development of free-boundary electrophoresis and analytical ultracentrifugation in the 1930s showed clearly that casein and the whey proteins are heterogeneous. This led to the development, during the 1950s, of methods to isolate homogeneous proteins. The principal whey proteins were crystallized about this time, but it has been impossible to crystallize the caseins, due to the lack of defined secondary and tertiary structures.

The introduction of zone electrophoresis in starch and especially polyacrylamide gels in the late 1950s showed that all the principal milk proteins occur in many isoforms, due to minor amino acid substitutions (genetic polymorphism), variations in phosphorylation and/or glycosylation and in some cases, intermolecular disulfide bonding and limited proteolysis. During the 1970s, the primary structure of all the principal milk proteins and the secondary, tertiary and quaternary structures of the whey proteins were determined.

The isolation of κ-casein in 1956 initiated research on the structure of the casein micelle, a process that continues with a series of refinements, made possible mainly through developments in electron microscopy and X-ray scattering. The stability of casein micelles is critical in most dairy products and processes, and aspects of stability and factors affecting it have been studied for more than a century, e.g., rennet-induced coagulation and stability to heat, concentration, dehydration, ethanol, homogenization or high pressure, the significance of which has varied over time and location.

The physico-chemical and biological properties of the milk proteins have been investigated

over a long period but especially since 1960. The caseins serve mainly as sources of amino acids and as carriers of calcium and inorganic phosphate, but all of the principal, and many of the minor, whey proteins have biological functions as well as serving as sources of amino acids. Since the 1960s, so-called 'functional' proteins have become increasingly important dairy products, and their physico-chemical properties, such as hydration, solubility, viscosity of their solutions, surface activity and gelation properties, have been characterized and modified by new technological processes. The introduction of membrane technology in the 1960s greatly facilitated the development of whey protein-based products which are now valuable dairy products and have converted whey from being a waste stream into a valuable dairy product.

Among the minor proteins of milk are about 70 enzymes, which originate mainly from the cytoplasm of the mammocytes, the MFGM, the animal's blood, through leaky junctions or leucocytes. The first paper on a milk enzyme (lactoperoxidase) was published in 1881, and since then there has been a continuous flow of research reports. The principal indigenous enzymes have been isolated and characterized, but many of the less important enzymes have been recognized only through their activity (for further information, see Chap. 12). The indigenous enzymes in milk are important for one or more of the following reasons: protective agents for the mammary gland or the neonate, in digestion, stability or spoilage of milk or dairy products, as indicators of milk quality, e.g., of mastitis, and especially as indicators/markers of milk treatment, especially heat treatment.

During the past 20 years, there has been considerable interest in the genetics and evolutionary aspects of milk proteins, especially of the caseins, and considerable progress has been made, including the structure of the milk protein gene cluster and elucidation of the synthesis and evolution of milk proteins. The caseins are members of a family of secretory Ca-binding phosphoproteins (SCPPs), which are believed to have evolved by gene duplication; other members are enamel matrix proteins and some salivary proteins. SCPPs are necessary for the mineralization of tissues which is considered to be a critical innovation for vertebrate evolution, forming the basis for various adaptations, including body armour for protection, teeth for predation and endoskeleton for locomotion. All SCPPs have many features in common and are believed to have evolved from a common ancestor (Kawasaki and Weiss, 2003; Kawasaki *et al*., 2004, 2011). The evolution of casein is considered to have been critical in the evolution of lactation and hence mammals (see Chap. 1). For more detailed information on this exciting aspect of dairy chemistry research, please see the following articles: Chanat *et al.* (1999), Peaker (2002), Rijnkels (2002), Lefevre *et al.* (2009) and Le Parc *et al.* (2010). It is very likely that research on the molecular biology of milk proteins will continue, and probably accelerate, in the immediate future and should be of great value to dairy chemistry.

Lactation is a characterizing feature of mammals, of which there are about 4,500 species. However, the milk proteins of only a few species (human, cow, sheep, goat, buffalo, pig, horse, donkey, camel, yak and mouse) have been characterized, even superficially. There has been interest in the interspecies comparison of the milk proteins for many years and has increased recently; it seems likely that comparative interspecies work on milk proteins will increase in the immediate future (for further information, see Chap. 13). Although the proteins of milk, especially of bovine milk, are now well characterized, there is still ample opportunity for research across the fundamental-applied spectrum on this important and interesting subject.

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Quantitation of Proteins in Milk and Milk Products

3

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Abbreviations

AOAC	Association of Official Analytical Chemists
Arg	Arginine
Asp	Asparagine
BSA	Bovine serum albumin
CD	Circular dichroism
CE	Capillary electrophoresis
CID	Collision-induced dissociation
CMP	Caseino-macropeptide
CN	Casein
COSY	Humonuclear shift correlation spectrometry
CZE	Capillary zone electrophoresis
dc	Derivative of the concentration
DE	Delayed extraction
DEAE-TSK	Diethylaminoethyl-TSK
DNA	Deoxyribonucleic acid
dr	Derivative of the response
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ES	Electrospray
ESI	Electrospray ionisation
FIA	Flow injection analysis
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared
Glu	Glutamine
HA	Hydroxyapatite
HI-HPLC	Hydrophobic interaction HPLC
His	Histidine
HPLC	High-performance liquid chromatography
IDF	International Dairy Federation
IEF	Isoelectric focusing
Ig	Immunoglobulin
IR	Infrared
ISO	International Standardization Organization
KN	Kjeldahl nitrogen
LC-MS	Liquid chromatography mass spectrometry
Lys	Lysine
MAD	Multiple anomalous dispersions
MALDI	Matrix-assisted laser desorption ionisation
Mid-IR	Mid-infrared
MIR	Mid-infrared
MLR	Multiple linear regression
MS	Mass spectrometry
NCN	Non-casein nitrogen
NIR	Near-infrared

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NMR	Nuclear magnetic resonance
NOE	The nuclear Overhauser effect
NPN	Non-protein nitrogen
PAGE	Polyacrylamide gel electrophoresis
PLG	Plasminogen
PLM	Plasmin
PLS	Partial least squares
PSD	Post-source decay
PTH	Phenylthiohydantoin
R	Reproducibility
R^2	Correlation coefficient
RP-HPLC	Reversed-phase HPLC
SD	Standard deviation
SDS	Sodium dodecylsulphate
SEC	Standard error calibration
SEP	Standard error prediction
Ser	Serine
SRID	Single radial immunodiffusion
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
Thr	Threonine
TN	Total nitrogen
TOCSY	Total correlation spectroscopy
TOF	Time-of-flight
TP	True protein
UHT	Ultrahigh temperature
UV	Ultraviolet
WPC	Whey protein concentrate
WPs	Whey proteins
α-La	α-Lactalbumin
β-Lg	β-Lactoglobulin
λ	Wavelength

3.1 Introduction

Compared with other food products, milk is a fairly simple fluid which has been studied thoroughly since the beginning of the nineteenth century. Its composition and the main characteristics of its various constituents are now well known. This is especially true for the amino acid sequences of its proteins. No other food product today has its proteins so well characterised. This makes protein analysis in raw milk fairly straightforward.

Protein analysis is certainly an important issue. In fact, the present basis for milk payment shows that proteins are now the most valuable constituent of milk, so the precision of their determination is critical. However, as soon as technological treatments have been applied, any quantitative measurement, except for nitrogen determination, becomes far more difficult. This is particularly true for protein denaturation, which is not a one-step phenomenon. In fact, for a given protein, denaturation leads to products that may differ according to the treatment, often with an ultimate transformation into insoluble aggregates. Furthermore, a number of chemical reactions may occur during the processing of milk, dairy products and non-dairy products that can lead to covalent modifications of proteins. This is important because with the use of membrane technology, which allows concentration of various milk protein fractions, milk protein can then be used extensively as an ingredient in a number of food products. Furthermore, the new area of development in functional foods and nutraceuticals has shown that some protein fractions and/or peptides are beneficial to the health of humans. This has led a great number of research laboratories to investigate methods for extracting specific peptides. Milk proteins have been hydrolysed intentionally to peptides and amino acids by proteinases and peptidases. Theoretically, the origin of any peptide with more than five amino acid residues, provided it can be isolated, can be established if it is derived from any milk protein. However, although the full characterisation of a milk protein hydrolysate is a difficult and time-consuming task, it is now performed routinely by a number of laboratories. The procedures that allow the determination of total or individual milk proteins in milk and dairy products will be reviewed as well as those used in non-dairy products. Finally, some applications of milk protein analysis in dairy and non-dairy products will be presented.

3.2 Definitions of Protein and Analytical Performance

Now, in almost every country with a highly developed dairy industry, protein content is the major constituent in milk quality payment schemes and breeding programmes. Natural variations in the concentration of milk proteins are large. Variation

depends on many factors, including breed, feed, country and regional factors, stage of lactation, condition of individual cows and seasonal changes. Thus, while the average normal concentration of protein in milk is said to range between 35 and 40 g L^{-1}, it may vary much more widely from one cow to the next (Marshall, 1995). It is normally accepted that proteins represent 95% of the nitrogen content in milk, the remaining 5% being non-protein nitrogen (NPN; urea, creatine, uric and orotic acids, peptides, ammonia, etc.) (Walstra, 1999). It is also recognised that ≈ 80% of the nitrogen of milk is attributable to the caseins and 20% to whey proteins and NPN.

3.2.1 Nitrogen Fractions in Milk

In milk, five fractions are currently analysed:

- Total nitrogen (TN)
- Non-casein nitrogen (NCN) = nitrogen content of soluble proteins and NPN obtained by an acid precipitation at pH 4.6
- Non-protein nitrogen (NPN) = non-protein nitrogen soluble in 12% TCA
- True protein (TP) = TN-NPN
- Casein protein (CP) = TN-NCN

3.2.2 Conversion Factors

The various milk proteins have specific amino acid sequences which are known for the major proteins. To estimate the amount of protein in milk and milk products, it is necessary to convert nitrogen into protein by multiplying the nitrogen content by a factor, called the Kjeldahl conversion factor. A value of 6.38 for this factor, originally proposed a century ago by Hammarsten and Sebelien, on the basis of the nitrogen content of 15.67% for purified acid-precipitated casein, is generally accepted and was confirmed in the latest IDF standard (IDF, 1993). However, this method for calculating the protein content raises two important questions. Firstly, the terminology "protein content" is not fully correct, since the proportion of NPN, within and between dairy products, varies from 3% to 8% in milk and up to 25–30% in whey. To avoid confusion, the term "crude protein" should be used to express the nitrogenous matter in milk. Its quantitative expression is represented by the amount of total nitrogen multiplied by 6.38 and is expressed as g per 100 g (or per kg or L) of milk or milk product.

Secondly, the conversion factor is not constant but is highly dependent on the amino acid

Table 3.1 Protein content of milk and Kjeldahl factor for milk (Karman and Van Boekel, 1986)

		Without carbohydrate		With carbohydrate	
Protein	Concentration (g/litre)	N%	Kjeldahl factor	N%	Kjeldahl factor
α_{s1}-Casein	10.0	15.77	6.34		
α_{s2}-Casein	2.6	15.83	6.30		
β-Casein	9.3	15.76	6.34		
κ-Casein	3.3	16.26	6.15	15.67	6.38
γ-Casein	0.8	15.87	6.30		
β-Lactoglobulin	3.2	15.68	6.38		
α-Lactalbumin	1.2	16.29	6.14		
BSA	0.4	16.46	6.07		
Ig	0.8	16.66	6.00	16.14	6.20
PP, 8 F, 8S	0.5	15.30	6.54		
PP3	0.3	16.97	5.89	15.27	6.55
Lactoferrin	0.1	17.48	5.72	16.29	6.14
Transferrin	0.1	17.00	5.88	16.10	6.21
MFGM	0.4	15.15	6.60	14.13	7.08
Milk	33.0	15.87	6.30	15.76	6.34

MFGM Milk fat globule membrane

Table 3.2 Experimental Kjeldahl factors for isolated milk protein (Karman and Van Boekel, 1986)

Protein	% Non-protein ash[a]	Corrected % N[b]	Experimental Kjeldahl factor	Theoretical Kjeldahl factor
α_s-Casein	2.16	15.55	6.43	6.33
β-Casein	3.78	16.41	6.10	6.34
κ-Casein	2.11	14.84	6.74	6.38
Total casein	1.40	15.62	6.40	6.34
β-Lactoglobulin	12.60	14.97	6.68	6.38

[a]The difference between the ash content and the sum of PO_4 and SO_4 contents taken as non-protein ash (for β-lactoglobulin only the SO_4 content was taken)
[b]Corrected for fat and water content and non-protein ash

composition of the protein fraction. Using the primary structure of milk proteins, Karman and van Boekel (1986) showed that for bovine milk, the conversion factor should be 6.34 instead of 6.38, and different factors should be used for casein (6.34), *para*-casein (6.29), proteins of rennet whey (6.45), acid whey proteins (6.30) and NPN (3.60). For individual proteins, the variability of the factor is even greater (Table 3.1). In their study, they demonstrated that experimental determination of the Kjeldahl factor on (pure) protein fractions leads to substantial discrepancies from the theoretical values calculated from amino acid sequences (Table 3.2), mainly because it is difficult to obtain pure fractions and to measure the ash content accurately.

3.3 Reference and Routine Methods

Nitrogen is the element that essentially characterises proteins in milk, as well as in other foodstuffs. The determination of nitrogen has always been used as a reference for estimating the protein content of foods.

3.3.1 Kjeldahl Method (Nitrogen Determination)

One of the most widely used methods for protein determination in foods is the Kjeldahl method. It is used to measure the nitrogen content of foods, which is converted to protein content by a conversion factor (see Sec. 3.2.2, "Conversion Factors"). This method is now internationally recognised as the reference method for measuring the protein content of milk products (IDF, 1993; AOAC, 1995) and is listed as such in the Codex Alimentarius.

Principle. In the Kjeldahl method, the organic compounds are digested in concentrated H_2SO_4 in the presence of a catalyst and perhaps an oxidising agent. The total organic nitrogen is converted quantitatively to $(NH_4)_2SO_4$, neutralised with NaOH, the NH_3 distilled off and estimated by titration with a standard acid. The result is multiplied by a conversion factor to give the crude protein content of the sample (Chang Sam, 1998). Detailed information regarding all the reactions involved in this procedure is given by Bradstreet (1965).

The total mineralisation time given in a standard method must be considered as a minimum time. The heating time should not be reduced if the clearing time (when the digest becomes clear) is short, as, for instance, with low-fat milk samples. On the other hand, for samples with a high fat or protein content, the amount of H_2SO_4 must be increased because organic material consumes H_2SO_4 and the total mineralisation time should be extended if the clearing time is longer than that given in the standard method. The latest version of the International Dairy Federation standard for Kjeldahl determination of milk proteins (total nitrogen and true protein content) is based on the results of two inter-laboratory studies, which involved ten laboratories (Barbano *et al*., 1990; Barbano and Lynch, 1991).

3.3.1.1 *Analysis*

Nitrogen (Total) in Milk (TN). The sample is digested without preparation. The amount of nitrogen is the total organic nitrogen (crude protein), which corresponds to the protein and non-protein nitrogen (IDF, 1993).

Non-protein Nitrogen (NPN). For a long time, TCA has been used to precipitate proteins in milk (Rowland, 1938). A solution of 15% TCA is added to milk to get a final concentration of 12%. This mixture is filtered; the filtrate contains the NPN compounds (urea, creatinine, creatine, amino acids and other minor nitrogen-containing compounds). True protein content is calculated from TN-NPN (Rowland, 1938). This procedure has been the subject of a collaborative study by Barbano and Lynch (1991) and is now a standard method (IDF, 1993).

True Protein (TP). The proteins are precipitated with 12% TCA, as for NPN, described above. The coagulum is collected on a filter and analysed directly for nitrogen content. This is one-step procedure, is faster and costs less than the two-step procedure (Barbano *et al*., 1990).

Non-casein Nitrogen (NCN). Milk caseins are defined as proteins that precipitate at pH 4.6 (Rowland, 1938). Lynch *et al*. (1998) conducted a collaborative study to modify and improve the current IDF procedure (IDF, 1964) for the determination of casein by the Kjeldahl method. The casein is precipitated at pH 4.6, using acetic acid and sodium acetate (Lynch *et al*., 1998). The NCN in the filtrate is measured by the Kjeldahl method (IDF, 1993). Casein content is calculated as the difference between total nitrogen and non-casein nitrogen.

Casein Nitrogen. The direct determination of casein is made by directly precipitating the casein at pH 4.6 in a Kjeldahl flask (Lynch *et al*., 1998), instead in an Erlenmeyer flask. This is done using 10% acetic acid and a sodium acetate solution (1 N). The casein precipitated is measured by Kjeldahl analysis (IDF, 1993). This method is also part of the revised IDF Standard 29 (IDF, 1999).

3.3.2 Infrared Methods

3.3.2.1 *Basic Principle of IR Measurements*

The IR Spectrum. Infrared radiation is electromagnetic energy longer than visible light and shorter than microwaves. Three regions characterise the IR spectrum according to the wavelength or wave number of the radiation: the near-IR region (NIR) from 0.7 to 2.5 μm (14,285–4,000 cm^{-1}), the mid-IR region (MIR), from 2.5 to 25 μm (4,000–400 cm^{-1}) and the far IR region from 25 up to 100 μm (400–100 cm^{-1}). The near and mid-infrared regions are the most useful for quantitative and qualitative analysis of foods. When a molecule is subjected to IR radiation, energy will be absorbed only if the frequency of the radiation corresponds to the frequency of one of the fundamental vibrations of the molecule (stretching vibrations at high frequencies and bending deformations at low frequencies).

The vibration energy which characterises a chemical group (e.g., C–H, O–H, C=O) is dependent on both the bond strength and the mass of the two atoms which form the group. Fundamental vibrations of molecules occur mainly in the MIR region and absorption in the NIR occurs at wavelengths which correspond to either harmonic frequencies or combination frequencies of the fundamental vibrations. These NIR absorption bands are generally quite broad, allowing the use of rather large spectral pass bands for measurements. Their intensities are weak, compared to the signals obtained in the MIR region from fundamental vibrations of the molecules, but they are sufficiently important to allow quantitative analysis. Both techniques have been used for the analysis of milk and milk products; MIR is used essentially for the analysis of milk or other liquid dairy products by transmission and NIR for the analysis of either liquid or solid dairy products by diffuse reflectance or transmittance. Rudzik (1985) compared the two techniques and concluded that they are complementary for the analysis of dairy products.

Quantitative Analysis. The IR energy absorbed by a sample can be measured either in the transmission mode, if the product is in solution or is sufficiently transparent to IR radiation, or in the reflection mode for opaque or solid samples. Most of the MIR instruments designed for liquid samples measure the transmitted light directly, while NIR instruments are usually built to measure the IR light which is either diffusely reflected

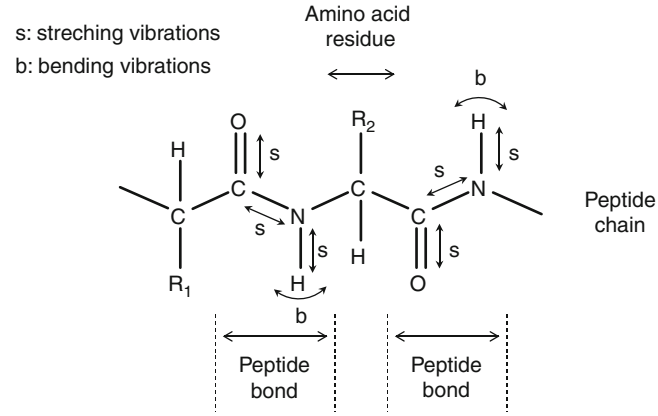

Fig. 3.1 Mid-IR absorption bands of a peptide bond: *a* amide I (C=O stretch), *b* amide II (C–N stretch), *c* amide II (N–H bending), *Left right arrow* direction of the light-induced dipole, *R1, R2* amino acid side chains

from the surface for solid samples or from the surface of liquids in a sample holder for liquids, or transmitted through a cuvette for liquids.

For a single-component solution, the amount of IR energy absorbed by the sample (absorbance) is exponentially proportional to the concentration of component and follows the Beer-Lambert law. If I_0 is the intensity of the incident beam and I is the intensity of the transmitted or reflected beam, the absorbance is $A_\lambda = \log I_0 / I = E_\lambda \cdot C \cdot l$, where the ratio I/I_0 represents either the transmittance or the reflectance, E the extinction coefficient of the component, C the concentration, λ the measurement wavelength and l the path length of the cell, for transmittance measurement.

The absorbance of n absorbing components is then expressed by the equation:

$$A_\lambda = (E_{\lambda 1} \cdot C_1 + E_{\lambda 2} \cdot C_2 + \cdots + E_{\lambda n} \cdot C_N) \cdot l$$

where $C_1, \ldots, C_N$ are the concentrations of the n components, and $E_{\lambda 1}$, $E_{\lambda 2}, \ldots, E_{\lambda n}$ are the extinction coefficients of the n components at the wavelengths $\lambda_1, \lambda_2, \ldots, \lambda_n$.

In conventional MIR instruments, to correct for any variations in the response of the system (source brightness, temperature of sample, soil on the cuvette, etc.) and to reduce, as much as possible, the influence of interfering components, like water, the measurements are made with reference to the amount of IR energy absorbed, either by water at the same wavelength as the assay wavelength, or by the sample at a nearby wavelength at which there is only a slight absorption by the component being measured. For NIR instruments using reflectance, the reference is usually obtained by the intensity of the incident beam reflected by a ceramic disk.

Analysis of Proteins by Mid-IR Spectroscopy. Today, almost all the milk payment, herd improvement testing and routine quality control are done using mid-IR analysers. Mid-IR analysis of proteins is now a standard method, referred to as IDF Standard 141B (IDF, 1996) and AOAC method 972.16 (AOAC, 1995). In the IDF monograph on indirect methods for milk analysis, Biggs *et al.* (1987) reviewed the available information regarding this technique. Most instruments are Fourier transform infrared (FTIR) spectrometers. Information on FTIR can be found in Van de Voort *et al.* (1992), Luinge *et al.* (1993) and in the reviews of Lefier (1998) and Agnet (1998).

Principle. There is a strong absorption band in MIR, called "amide II," at ~6.46 μm (1,550 cm^{-1}) by a peptide bond. This absorption originates from the C–N stretching vibration (40%), and from the N–H bending deformation (60%) (Fig. 3.1). The peptide bond also shows other absorption bands near 1,650 cm^{-1} (6.1 μm, "amide I," due mainly to C=O stretching vibration) and at 3,300 cm^{-1} (3.0 μm, N–H stretching vibration). As illustrated in Fig. 3.2, the determination of protein concentration in milk is based on the "specific" absorption of the peptide linkages at 6.46 μm. Although proteins are the major absorbing compounds at this wavelength, the absorbance is influenced by the

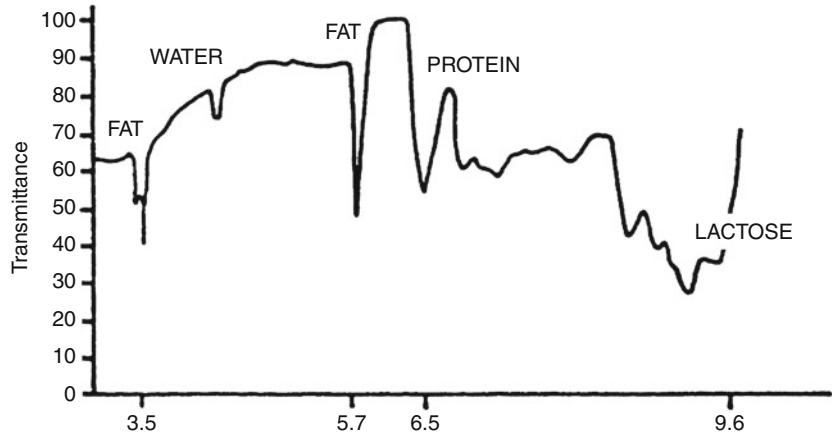

Fig. 3.2 The infrared transmission spectrum (wavelength in μm) of milk versus water (courtesy of Multispec Ltd; IDF, 1987)

other major compounds in milk (fat and lactose) and by minor soluble constituents. An automatic correction is achieved by setting internal intercorrection factors for each component at each wavelength. Intercorrection factors can be determined either with specifically prepared samples to characterise each of the intercorrection factors or by multiple regression of uncorrected instrument signal versus chemical data on a large number of milk samples (Barbano and Clark, 1989). The poor resolution of the spectra has been enhanced by the data treatment algorithms to reduce the strong absorption band of water.

Analysis. A warmed sample (40°C), thoroughly mixed, and if necessary, blended and/or diluted, is pumped through a 1-, 2- or 3-stage valve homogeniser. Before analysis, the instrument must be calibrated using a reference method. Natural milks and reconstituted milk (from raw milk, cream, skim milk, retentate and ultrafiltrate) are the only two types of calibration accepted according to IDF (1996).

Instrumentation. FTIR instruments are equipped with a single cell. They also include a source of polychromatic beam emission and a Michelson interferometer to split the polychromatic beam. The interferometer uses a beam splitter to divide the incident polychromatic radiation (source) into two parts, each reflected to a fixed and a moving mirror, respectively. The divided beams are recombined at the beam splitter by reflecting them back with mirrors. Because of the moving mirror, the two beams undergo constructive and destructive interference as they recombine at the beam splitter. Intensity fluctuations produced by the interference are measured by the detector, digitised in real time and referred to an interferogram. A mathematical treatment, called the Fourier transform, is used to convert the resulting interferogram into a typical IR spectrum. FTIR instruments offer significant advantages over dispersive spectrometers: While providing a great improvement of the signal-to-noise ratio, FTIR instruments also detect all the wavelengths simultaneously and therefore acquire spectra more rapidly. Figure 3.3 illustrates an FTIR instrument.

3.3.2.2 Factors That Affect the Accuracy of Mid-IR Protein Determination in Milk

Physicochemical Factors. Aside from the influence of instrumental factors (for complete information, see IDF, 1996), like temperature, linearity (Smith *et al*., 1993a), water vapour in the optical console, homogenisation (Smith *et al*., 1993b; Smith *et al*., 1995), etc., and assuming that the instrument is correctly calibrated (including the correction for fat and lactose), the accuracy of milk protein testing is influenced mainly by variations in the proportion of NPN and by the presence of carboxylic acids. The relationship between IR absorption at 6.46 μm and true protein concentration, measured by Kjeldahl, is relatively independent of the amino

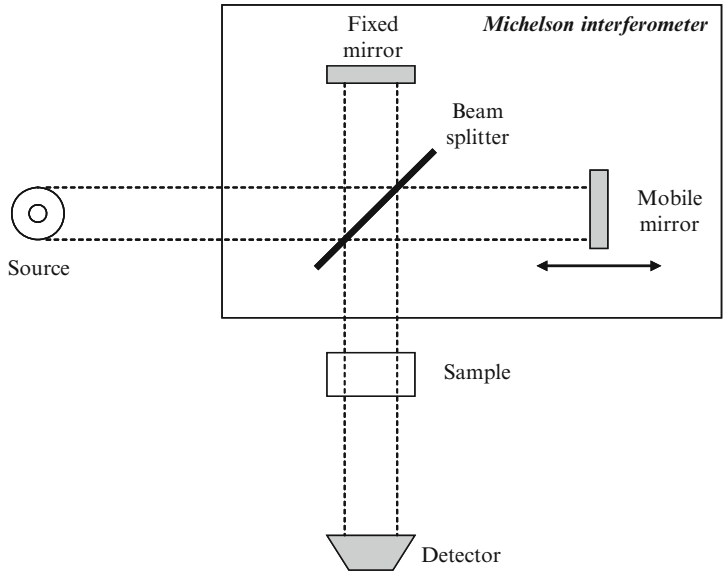

Fig. 3.3 Schematic representation of the principle of Fourier transform (FTIR) spectrometer based on a Michelson interferometer. From Lanher (Lanher, 1996), *J. Assoc. off. Anal. Chem. Int*., Vol. 79 (6). S: source, D: detector

acid composition of the protein, since the ratio, N content/number of peptide bonds, is relatively constant. On the other hand, because the NPN fraction is not measured by IR, any variation in the proportion of NPN will influence the accuracy of an instrument calibrated to measure crude protein (total N×6.38).

Ionised carboxyl groups, COO^-, absorb at the protein absorption wavelength. The main indigenous source of such groups in milk is citrate. Sjaunja and Anderson (1985) have shown that natural variations in the citrate content of individual milks explain 40–60% of the difference between the IR and the Kjeldahl true protein results and an increase of 0.01 g/100 g of milk in the concentration of citric acid increases the protein reading by 0.075 g/100 g.

The formation of carboxylic acids on fermentation of lactose may also cause interference absorption at the protein wavelength (Goulden, 1964). Grappin and Jeunet (1979) clearly demonstrated that, in fact, most of the interfering compounds are present in the soluble phase of milk (Fig. 3.4).

Biological Factors. Any biological factor (e.g., stage of lactation, mastitis, breed, species, feeding, season) known to influence one of the physico-chemical characteristics mentioned above will, in turn, cause systematic errors in protein measurements by IR methods. According to Biggs *et al*. (1987), the influence of only a few factors has been demonstrated clearly. Goats' milk, which has a lower citrate concentration than bovine milk, requires a different calibration for true protein analysis than those for cows' milk (Grappin *et al*., 1979). Season/feeding, as well as species (goat vs cow) or breed (Jersey vs others), which influences the proportion of NPN in milk, will have a significant influence on the accuracy of the method when the apparatus is calibrated for crude protein (Grappin and Jeunet, 1979). Whenever possible, adjustments of the instrument calibration will, therefore, have to be made.

Casein Determination. Sjauna and Schaar (1984), Karman *et al*. (1987) and Barbano and Dellavalle (1987) performed a two-step determination of casein (milk and filtrate containing whey or

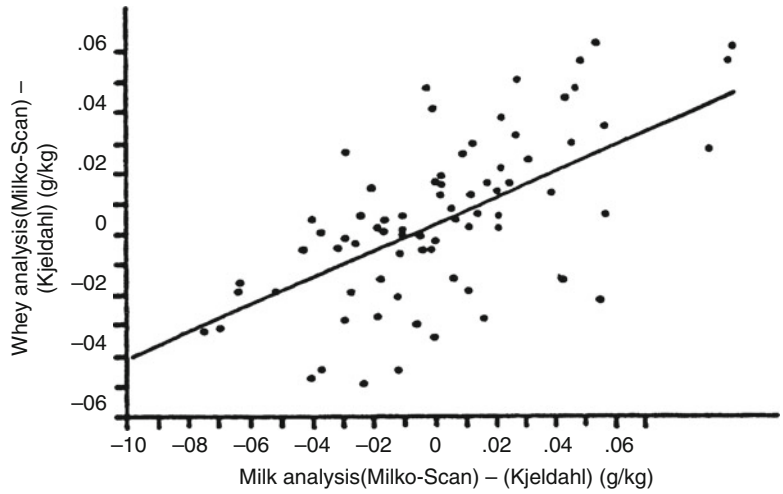

Fig. 3.4 Relationship between MilkoScan protein readings-true protein Kjeldahl values, for 81 individual goat milk samples (*x*) and the corresponding whey samples (*y*) (Grappin and Jeunet, 1979). Coefficient of correlation, r = 0.65

non-casein protein) and, by subtraction and usually after the application of correction factors, obtained the casein content. Using an FTIR analyser, Hewavitharana and Brakel (1997) determined the casein concentration in raw milk directly. They obtained a mean difference from the reference method of 0.4% and a correlation coefficient of 0.976. Casein determination by mid-IR is not an official standard, but with modern FTIR instruments, it is now used routinely for analysis of milk.

Application to Dairy Products. To analyse viscous or semi-solid products with a high protein content, dilution and vigorous blending of a weighed sample is necessary before analysis using the commercial instruments. Because of the interference by other compounds and the influence of the technological process, specific instrument calibration is necessary for each product. Moreover, better results will be obtained before any hydrolysis of the proteins occurs. Although MilkoScan or Multispec milk analysers are commonly used by the dairy industry for quality control of their products, little information is available on the analytical performance of the method.

Analysis of Proteins by NIR Spectroscopy. In 1985, NIR calibrations were developed for the principal constituents of dairy products, and it was recognised that the NIR technology fulfilled nearly all the current official analytical performance requirements. The next few years were characterised by developments in software, hardware, grating monochromators and by improvements in optical and electronic components. In the same way, the development of chemometry led to new complex NIR applications in both transmittance and reflectance modes. The dairy industry is now increasingly using NIR methods to monitor the quality of dairy products, for example, moisture content of milk powder and moisture, protein and fat content of milk, curd and yogurt. Despite all those applications, NIR spectroscopy is still not an official method of analysis in the dairy industry. For detailed information concerning theoretical aspects, instrumentation, calibration and application of NIR spectroscopy in food analysis, the textbook of Osborne et al. (1993) should be consulted. For more specific information on the dairy industry, Rodriguez-Otero et al. (1997a) and Laporte and Paquin (1998a) published reviews on the use of near-infrared spectroscopy for the analysis of dairy products.

Principle. The NIR region of the electromagnetic spectrum (700–2,500 nm) includes molecular absorptions of overtone (700–1,800 nm) and combination bands (1,800–2,500 nm). Wavelength (λ)

units (nm) are often used in the NIR region of the electromagnetic spectrum but can also be expressed as wave numbers (cm^{-1}), which equal 10,000/λ (λ in μm) or $10^7/\lambda$ (λ in nm). Covalent bonds involving hydrogen (C–H; N–H and O–H) are dominant in the NIR region. NIR band intensity is weaker (by a factor 10–100) than their corresponding MIR bands. Spectra can be collected either in reflectance or transmittance (usually preferred with liquids) modes. Because of the strength of the overtones (1,450 nm) and combination (1,940 nm) water bands in the NIR region, milk has an NIR spectrum very similar to that of water. Spectral bands related to the other milk components are difficult to isolate from the raw (unprocessed) spectrum. Furthermore, milk spectra result from the sum of each milk component and their specific interactions. Determination of wavebands that are specific to milk proteins can be achieved only by mathematical processing of a collection of milk spectra. However, the derivative mathematical treatment is an alternative approach to the problem of overlapping peaks (Hruschka, 1987). In the second derivative of average milk spectra, the characteristic absorption peaks are more clearly separated (Fig. 3.5a, b). The lipid C–H combination and second overtone can be seen at 2,320 and 2,350 nm (Giangiacomo and Nzabonimpa, 1994). The absorption of N–H structures related to protein are located at approximately 2,060 and 2,170 nm (Diaz Carillo *et al*., 1993). Table 3.3 gives an example of the wavelengths that have been assigned by different authors for NIR protein analysis.

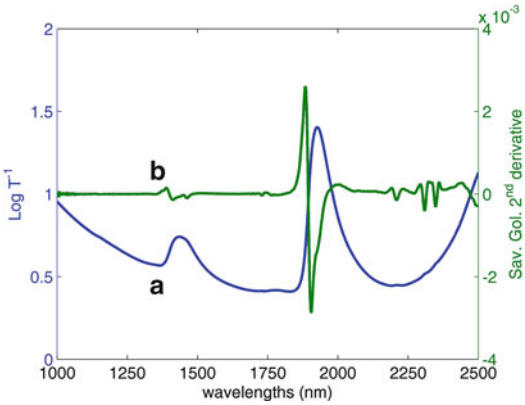

Fig. 3.5 Near-infrared spectrum of milk: (**a**) raw spectrum (**b**) second derivative. Courtesy of T.M.P. Cattaneo (CRA-IAA, Milan) and S. Barzaghi, (CRA-FLC, Lodi); CRA - Consiglio per la ricerca e sperimentazione in Agricoltura – Italy

The NIR analyser must be calibrated prior to any protein measurement. Calibration equations quantify the relationship between the NIR absorption information and the laboratory reference method. The accuracy of this relationship is measured with the standard error of calibration (SEC) and the standard error of prediction (SEP).

Sample preparation varies according to the nature of the product. For milk analysis, after homogenisation to limit the light scattering effect of fat globules, the sample is usually placed into a temperature-controlled (40 ± 0.1°C) holder with a quartz window. Powder samples

Table 3.3 Waveband assignments (nm) for NIR protein analysis of dairy products

Dried milk	Liquid milk	Dried milk	Casein and dried cheeses	Liquid milk	Liquid milk	Cheddar cheese curd
Goulden (1957)	Jeunet and Grappin (1985)	Baer *et al.* (1983)	Frank and Birth (1982)	Robert *et al.* (1987)	Kamishikiryo-Yamashita *et al.* (1994)	Lee *et al.* (1997)
1,180			1,170			
			1,290			
1,450–1,600	1,450		1,500			
1,730		1,730	1,700			
1,820	1,820	1,820				
1,930		1,980				
	2,100			2,050		
3,050	2,180	2,190		2,180	2,170	2,138
2,280						2,287
2,320		2,310				

Table 3.4 Principal suppliers of mid-IR and near-IR instruments for analysis of milk and dairy products

Mid-infrared			Near-infrared		
Manufacturer	Instrument	Wavelength selection system	Manufacturer	Instruments	Wavelength selection system
Foss Electric A/S 69, Slangerupgade DK-3400 Hillerroed, Denmark	MilkoScan FT 120 MilkoScan FT1 MilkoScan FT+ MilkoScan Minor	FTIR FTIR FTIR Filters	ABB Bomem Inc. 585 Blvd Charest suite 300, Quebec (Qc), Canada, GIK 9 H4	Network*ir* Formulat*ir* Butter and Margarine analyser	FTIR
Bentley Instruments Inc. 4004, Peavey Road, Chuska, MN, 53318, USA DeltaInstruments B.V. Kelvinlaan 3 9207 JB Drachten, the Netherlands	Bentley FTS DairySpec FT Bentley 150 Bentley 2000 LactoScope FTIR LacoScope Filters	FTIR FTIR Filters Filters FTIR Filters	Brucker Optics Inc. 19, Fortune Drive, Billerica, MA, 01821–3991, USA	MPA	FTNIR
			Bran + Luebbe Inc. 1025, Busch Parkway, Bufflo Grove, IL, 60089–4516, USA	InfraAlyzer series	Filters
			Dickey-john Corp. 52000 Dickey-john Road, Auburn, IL, 62 615, USA	Instalab and GAC III	Filters
			Leco Corp. 3000 Lakeview Av, St. Joseph, MI, 48085–2396, USA	Quick-Chek series	Filters
			LT Industries Inc. 6110 Executive Bvld # 200, Rockville, MD, 20852, USA	Quantum series	Grating
			FossNIRSystems 12101, Tech. Road, Silver Spring, MD, 20 904, USA	NIR series	Grating
			Perkin-Elmer Corp. 761 Main Avenue, Norwalk, CT, 06859, USA	Spectrum one	FTIR

are simply placed in a sample holder and pressed against a quartz window. Solid or pasty products, like cheese, should have a uniform surface and are placed in open holders or other plastic devices. To obtain reliable results, sample preparation is extremely important. For protein measurement, de Vilder and Bossuyt (1983) pointed out that the granular structure of milk powder affects the results.

Analysis and Instrumentation. All NIR instruments have five basic parts: a radiation source (a tungsten-halogen lamp in most NIR instruments), a wavelength dispersion device, a detector (usually, lead sulphide or silicon) and finally many electronics components and a computer. As for the selection wavelength device, essentially three types of instrumentation are available on the market for food analysis (Table 3.4): filter (tilting or fixed),

monochromator, which consists of a grating device that scatters the incident polychromatic beam in a series of diverging monochromatic beams, and Fourier transform instruments. Contemporary NIR spectrophotometers are of the two last types.

Analytical Attributes and Factors That Affect the Accuracy of Protein Testing in Milk. With the exception of instrumental and sample factors, compared to MIR techniques, little work has been done to assess the performance of the NIR instruments for the analysis of milk and to evaluate thoroughly the physicochemical and biological factors that may influence the response of NIR analysis. On individual samples of cows' and goats' milks, Jeunet and Grappin (1985) found that lipolysis did not interfere and that only the species of animal had a significant effect on protein results. Conversely to MIR, the accuracy SD is slightly lower (0.021 vs 0.025 g/100 g) when the instrument is calibrated for crude instead of true protein. Similarly, a better estimate was obtained by Baer *et al*. (1983) on non-fat dry milk when the Kjeldahl method was used as a reference rather than the dye-binding method.

Casein Determination. Sato *et al*. (1987) first investigated the feasibility of casein determination by MLR (SEC = 0.0951 and $R^2 = 0.854$) using NIR reflectance. Kamishikiryo-Yamashita *et al*. (1994) added more theoretical work on the subject. Diaz-Carillo *et al*. (1993) presented calibrations for goat milk casein and casein fractions. The sample was dried on glass fibre filters and the SEP = was 0.35 for total casein. Finally, Laporte and Paquin (1999) performed casein calibrations (SEP = 0.06) with NIR transmittance spectroscopy on cows' milk.

Application to Dairy Products. NIR spectroscopy is widely used for fat, protein, lactose and dry matter determination in milk and cream. NIR techniques have also been evaluated for the analysis of moisture, fat and protein in various milk powders (Baer *et al*., 1983; de Vilder and Bossuyt, 1983; Egli and Meyback, 1984; Frankhuizen, and van der Veen, 1985), cheese (Frank and Birth, 1982; Frankhuizen and van der Veen, 1985; Wehling and Pierce, 1994; Rodriguez Otero *et al*., 1995; Lee *et al*., 1997), and fermented milks (Rodriguez Otero and Hermida, 1996; Rodriguez Otero *et al*., 1997b). Finally, NIR spectroscopy was used successfully for monitoring the rennet coagulation of milk (Payne *et al*., 1993; Saputra *et al*., 1994; Laporte and Paquin, 1998b) and whey protein denaturation (Pouliot *et al*., 1997).

3.4 Separate Determination and Characterisation of Individual Proteins in Milk and Dairy Products

Each protein has specific physicochemical properties that determine the overall characteristics (technological, nutritional and sensory properties) of the food products they are involved in. Analytical methods that provide quantitative and qualitative information on proteins (e.g., protein content, genetic variants, degradation products of proteins, etc.) and interaction between proteins in a mixture (raw milk, dairy products) are of paramount importance to evaluate their behaviour during processing and digestion. These analytical methods are also used for the detection of adulteration and to evaluate protein modifications at molecular or supramolecular levels occurring during storage.

The main techniques that have been used to quantify the main proteins in milk and other dairy products are electrophoresis, column liquid chromatography and immunochemical methods. Using electrophoresis and column liquid chromatography, proteins are separated from a mixture prior to quantification while immunochemical methods give direct quantification. Structural information on proteins is provided using spectroscopic methods: Circular dichroism (CD) and infrared (IR) spectroscopy are the main methods used to acquire information on the secondary structures of proteins. NMR spectroscopy and X-ray crystallography are used for the determination of the three-dimensional structure of the proteins. Mass spectrometry (MS) permits the identification and quantification of proteins and degradation products of proteins even in trace

amount. MS has led to substantial progress in the characterisation of milk proteins, with major emphasis on the determination of new genetic variants, on post-translational and chemical modifications. Matrix-assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI) are currently the dominant methods for ionisation of biomacromolecules, such as proteins.

3.4.1 Electrophoresis

(a) *Native Electrophoresis*

This technique has been widely used for phenotyping individual cows and for determining the main proteins in raw milk. The term "native" electrophoresis refers to the analysis of the protein under native-like conditions, compared to other forms of electrophoresis, where denaturing (SDS or urea) or reducing agents (mercaptoethanol or dithiothreitol [DTT]) are used, see later sections. Electrophoresis is performed mainly using polyacrylamide gels as the separating matrix. However, starch or polyacrylamide-agarose mixtures have also been used successfully for separating caseins (Wake and Baldwin, 1961). Most gels are run in a discontinuous buffer system, in which proteins migrate as discrete bands, due to the use of discontinuous buffers and a gel system involving a stacking gel as a layer above the separation gel (Andrews, 1986). Further improvements in gel electrophoresis have been obtained through the use of thin mini-gels (5×4 cm, 0.4–1 mm thickness) and silver staining, which have increased both resolution and the sensitivity of the technique and reduced the time of analysis considerably. In addition, the commercial availability of precast mini-gels and semiautomated electrophoresis equipment makes it possible to resolve, stain and destain proteins in a few hours (van Hekken and Thompson, 1992). The development of the gel electrophoresis technique, its evolution and application to protein analysis in milk or dairy products have been extensively reviewed by Shalabi and Fox (1987), Creamer (1991) and McSweeney and Fox (1997). The technique has been performed on milks for genetic purposes but also for monitoring and assessing protein breakdown during the storage or processing of milk or for detecting adulteration, as reported by Strange *et al.* (1992). The techniques are identical to those mentioned above, and densitometric scanning is required. Separate electrophoresis at alkaline pH values is performed on casein precipitate obtained at pH 4.6 (which are then dissolved with urea and a reducing agent) and whey. When vertical slab gels are used, *para*-κ-CN, which can often be found in milk at small concentrations, cannot be determined since it migrates upwards at alkaline pH. Amido Black 10B or Coomassie Blue G250 is used to stain proteins. To determine the absolute amount of proteins following scanning densitometry, which gives areas proportional to the amounts, it is necessary to know their dye-binding capacities. On electrophoresis, α_{s1}-, α_{s2}- and κ-CN Indices bands give 2, 4 and 1 major and several minor bands, respectively. The several κ-CN bands are transformed by chymosin into one (or two) *para*-κ-CN band(s). When κ-CN is determined directly on vertical gels (without chymosin treatment), only the major band is considered.

(b) *SDS Electrophoresis*

SDS binds strongly to proteins, mainly through hydrophobic interactions. The amount of SDS bound is approximately proportional to the weight of the protein: ~1.4 g SDS/g protein (Reynolds and Tanford, 1970). Thus, any protein molecule will bind a large number of SDS molecules, each of which carries a negative charge of the sulphate group of SDS. The indigenous net charge on the protein at any pH is thus made negligible. Therefore, all proteins should, in the presence of SDS, migrate at the same velocity towards the anode in free-flow electrophoresis. However, in zone electrophoresis, particularly in acrylamide gels, the larger the protein, the lower its electrophoretic mobility, because of the sieving action of the gel.

This technique is used widely to determine the molecular weight, MW, of proteins (Shapiro *et al.*, 1967) with the possibility of covering a wider range of MW values by

Table 3.5 Isoelectric point of milk proteins determined by isoelectric focusing

Protein	Interval at probability level of 5%		
	Erhardt (1993)	Seibert *et al*. (1985)	Trieu-Cout and Gripon (1981)
α_{s1}-CN A	–	4.16–4.40	–
B	–	4.23–4.47	4.44–4.76
C	–	4.27–4.49	–
α_{s2}-CN A	–	7.83–5.13	–
D	4.68–4.96	–	–
β-CN A1	–	4.68–4.96	–
A2	–	4.60–4.84	4.83–5.07
A3	–	4.50–4.74	–
B	–	4.78–5.10	–
C	–	4.97–5.29	–
κ-CN A	–	5.43–5.81	5.45–5.77
B	–	5.54–6.12	–
C	5.83–5.62	–	–
α-La B	–	4.66–4.90	–
β-Lg A	–	4.64–4.90	–
B	–	4.72–4.98	–
C	–	4.77	–
γ1-CN	–	–	5.55–5.87
γ2-CN	–	–	6.38–6.72
γ3-CN	–	–	6.01–6.29

[a]Correlation for pH shift due to urea was made by subtracting the pH difference between an ampholyte solution and the same solution plus urea, ampholytes and urea being at the same concentration as in the gel

varying the pore size and by using an acrylamide concentration gradient (Rodbard *et al*., 1971). The most widely used method for SDS-PAGE gels of both casein and whey proteins is that of Laemmli (1970) under both reducing and non-reducing conditions (presence or absence of mercaptoethanol or DTT). The four bovine caseins can be separated by SDS-PAGE in the presence of a reducing agent (Creamer and Richardson, 1984), giving four distinct bands corresponding, in the order of increasing mobility, to α_{s1}-, α_{s2}-, β- and κ-CNs. It was noted that α_{s1}- and β-CNs behave atypically, giving higher MW values (Creamer and Richardson, 1984). Furthermore, the following proteins, each having a mobility higher than those of the caseins, can also be distinguished by SDS-PAGE of milk: γ_1-CN, β-Lg, α-La + *para*-κ-CN, γ_2- + γ_3-CNs, in the order of increasing mobility. This method could give, by scanning densitometry of a single gel, the absolute amounts of the main proteins present in individual or bulk milks.

(c) *Isoelectric Focusing (IEF)*

Trieu-Cuot and Gripon (1981) clearly identified the different proteins in whole casein by using IEF. The separation was performed in 1 mm-thick polyacrylamide gels containing ampholytes, 7 M urea and 0.1% 2-mercaptoethanol. The following components were identified, in order of decreasing isoelectric pH value: γ-CNs (the 3 known components), κ-CN (2 components), α_{s2}-CN (the 4 known components), β-CN and α_{s1}-CN (several components, including α_{s0}). IEF was used for phenotyping, in a single run, all milk proteins in ultrathin-layer polyacrylamide gels. The following variants were detected: α_{s1}-CN A, B, C: α_{s2}-CN A, D; β-CN A^1, A^2, A^3, B, C; κ-CN A, B, C; α-La B; β-Lg A, B, C. Table 3.5 gives the isoelectric pH values that have been measured (Trieu Cuot and Gripon, 1981; Erhardt, 1993). Rapid identification of the genetic

variants of milk proteins using the PhastSystem (Pharmacia) was reported by Vegarud *et al.* (1989) who analysed the caseins (10% Servalytes, pH 4.0–6.0, 4.5–5.0, 5.0–7.0, 1/1/1, v/v/v; 8 M urea, 2.5% Triton X-100, and 2-mercaptoethanol + urea in the samples) and whey proteins (Phastgel IEF, pH 3–9 without urea) separately in 350 individual cow milk samples. All the variants mentioned above were detected. Another widely used procedure, developed by Bovenhuis and Verstege (1989), permits separation of all the milk proteins on a single gel (16% ampholytes, pH 4.2–4.9, 4.5–5.4, 3.5–5.0, 1/1/1, v/v/v; 8 M urea/0.8% Triton X-100/ 2-mercaptoethanol + urea in the samples of whole milk). The genetic variants mentioned above produced sharp distinct bands except for α-La B and β-Lg A, which co-migrated. This method can be used routinely for milk phenotyping.

(d) *Two-Dimensional Electrophoresis*
This technique is especially useful in the qualitative analysis of complex mixtures of proteins by taking advantage of two different criteria simultaneously; the isoelectric pH (or electrophoretic mobility) and the MW. Trieu-Cuot and Gripon (1981) separated caseins using IEF (pH 4–9) in the first dimension and PAGE (from 1 to 28% acrylamide) in the presence of 0.1% SDS and 4.9 M urea in the second dimension. Miranda (1983) separated the caseins, *para*-κ-CN, β-Lg and α-La in the 12% TCA (insoluble fraction of UHT milks). Significant progress has been made on the reproducibility of separation by using commercial strips with ampholytes immobilised in an acrylamide gel (Immobiline technique) (Gorg *et al*., 1995).

(e) *Capillary Electrophoresis (CE)*
Most forms of classical electrophoresis, including zone electrophoresis, isoelectric focusing and gel electrophoresis, have now been performed in the capillary format. CE offers significant advantages over traditional electrophoresis, including on-line detection by coupling with spectroscopic detectors to enhance sensitivity and to obtain quantitative data. The method gives high resolution with a number of theoretical plates as high as 10^6, with low solvent consumption and small sample requirements. It can be automated for large-scale routine application. For an overview of CE technique and application of dairy proteins, the reader is referred to reviews by Lindeberg (1996), Dong (1999) and Recio *et al*. (1997a, b, c).

(f) M*icrofluidic "Lab-on-a-Chip" Techniques*
The past decade has seen a rapid development in microfluidic techniques, including those for electrophoresis. While the technique has been widely adapted for DNA analysis, progress has been somewhat slower for protein separation. Anema (2009) directly compared the method with SDS-PAGE: αs-la, β-lg, αs-CNs, β-CN and κ-CN were readily separated in a milk system. However, the Ig's, Lf and BSA could not be resolved from the background in the microfluidic chip technique, but were easily resolved by SDS-PAGE. In the study, up to ten samples at once were analysed within 30 min, which is a major advantage of the techniques, though the relative standard error has been reported to be as high as 15%, indicating that this method is not yet sufficiently reproducible for routine quantitative protein analysis.

3.4.2 Column Chromatography

In the analytical field, high-performance chromatography has essentially replaced conventional liquid chromatography. Most of this section will focus on HPLC methods. Now, progress on methodologies is observed primarily in the improvement of stationary phases (chemical nature, pore size, stability, bead size, pore/shell structure…), enhancing the resolving power of the columns and reducing the time of analysis. Extensive reviews describing the analysis of most dairy products by HPLC and FPLC have been published by Gonzalez-Llano *et al*. (1990) and Strange *et al*. (1992).

(a) *Gel Filtration*
Although several attempts have been made to improve separation on high-performance gel

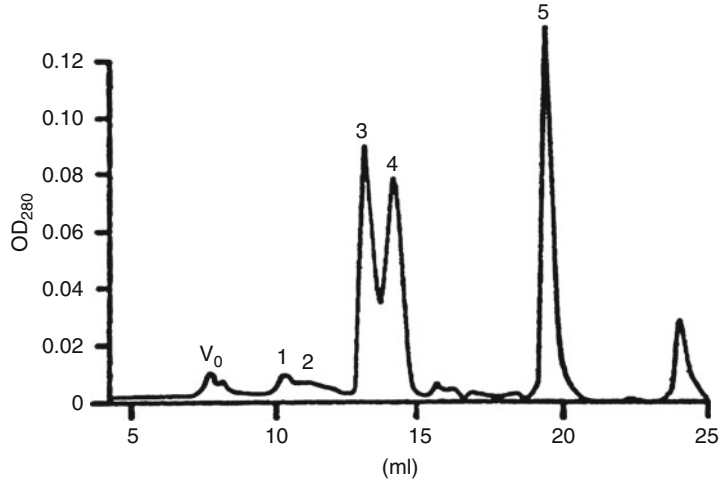

Fig. 3.6 FPLC separation of whey proteins by gel filtration on a Superose 12 column (Andrews *et al*., 1985). Fifty microlitres of fresh acid whey injected; flow rate 0.5 ml/min; eluent 0.1 M Tris–HCl buffer pH 7.0 containing 0.5 M NaCl and 10 mM NaN_3. *1* Ig's, *3* β-Lgs, *4* α-La, *5* orotic acid

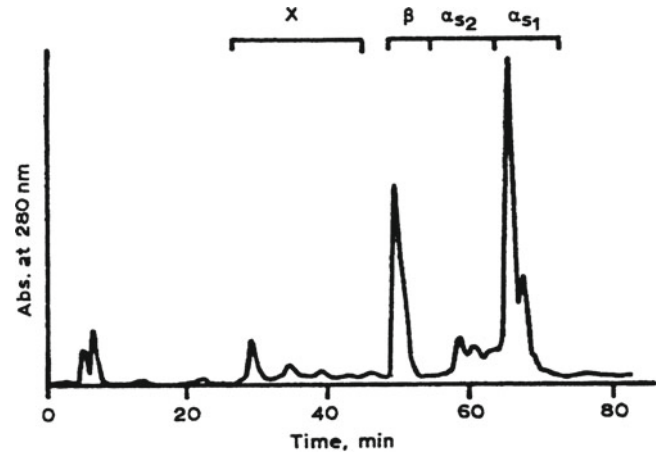

Fig. 3.7 FPLC separation of whole casein by anion exchange on a DEAE-TSK-5PW column (Aoki *et al*., 1987). Conditions are given in the text. Column size: 7.5×75 mm

filtration columns (Dimenna and Segall, 1981; Shimazaki and Sukegawa, 1982; Gupta, 1983), this method has not until now been suitable for analysing skim milk or whole casein. In contrast, whey proteins are separated quite well by this method, which permits their quantitation (Dimenna and Segall, 1981; Shimazaki and Sukegawa, 1982; Gupta, 1983; Humphrey, 1984; Andrews *et al*., 1985). Andrews *et al*. (1985), using a Superose 12 column (GE Healthcare), obtained separation suitable for the quantitative analysis of the main whey proteins (Fig. 3.6).

(b) *Ion Exchange*

No satisfactory result has been obtained on directly fractionating total milk proteins using ion exchange. However, both whole casein and whey proteins can be separated under conditions suitable for quantitative analyses.

Numerous studies report the separation of reduced non-alkylated whole casein by HPLC on anion exchangers (Humphrey and Newsome, 1984; Visser *et al*., 1986; Guillou *et al*., 1987). Excellent separation of previously reduced whole casein was obtained by Aoki *et al*. (1987) using a DEAE-TSK-5PW column with a 0.02 M imidazole buffer (pH 8.0) containing 3.3 M urea and 80 mM NaCl (Fig. 3.7). Based on the peak area and the extinction coefficients of each protein, the following proportions (means of two determinations), expressed as percent of whole casein, were obtained: α_{s1}-CN: 38.2; α_{s2}-CN: 11.0; β-CN: 39.5; κ-CN: 11.3. Figure 3.8 shows the

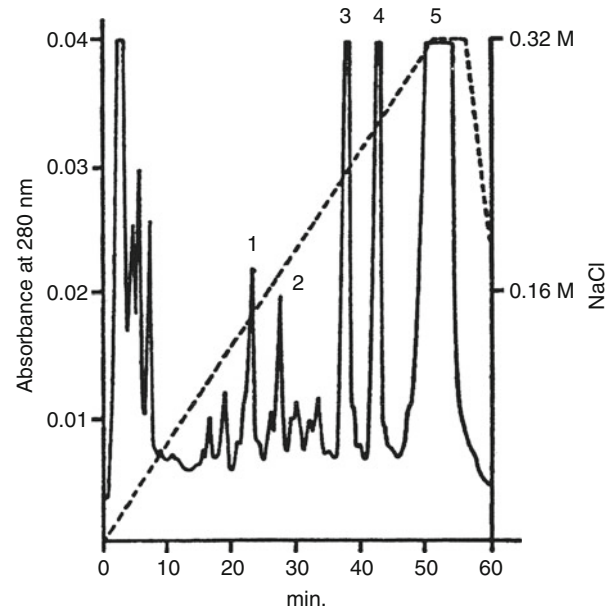

Fig. 3.8 FPLC separation of individual whole casein (κ A/B, β C/A^1, α_{s2} A, α_{s1} B) by anion exchange on a Mono Q column (Guillou *et al*., 1987). Casein sample in 5×10^{-3} M Tris–HCl$^-$, 4.5 M urea buffer pH 8.0, -8×10^{-4} M dithiothreitol; flow rate: 1 mL/min; 40°C; elution with the same solution as above with a 0–0.32 M NaCl gradient. *1* κ_0-caseins, B, *2* κ_0-caseins A, *3* β-caseins C, *4* β-caseins A^1, *5* α_{s2}-, α_{s1}-, α_{s0}-caseins. Overloading allows clear visualisation of the κ-caseins reactions, but decreases resolution between α_{s2}- (left part of the main peak) α_{s1}- (main peak), α_{s0}- (right part of main peak) caseins

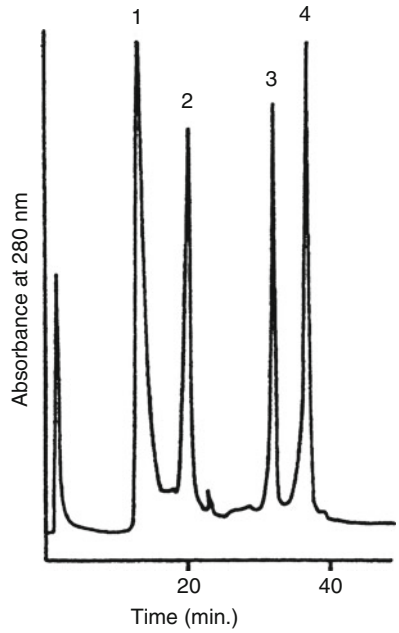

Fig. 3.9 FPLC separation of acid whey by anion exchange on a Mono Q column (Humphrey and Newsome, 1984). Acid whey sample; flow rate: 0.5 mL/min; elution in 0.02 M piperazine buffer, pH 6.0, with a 0–0.4 M NaCl gradient. *1* Orotic acid, *2* α-lactalbumin , *3* β-lactoglobulin B, *4* β-lactoglobulin A

separation of an individual casein sample on a Mono Q column in the presence of both urea and a reducing agent. In this case, some genetic variants were separated. Guillou *et al*. (1987) separated κ-CN A and B, and β-CN A^1, B and C by using a Mono Q column. Using the same ion exchanger, Dalgleish *et al*. (1985) resolved κ-CN A and B. Although the cation exchanger gave poorer resolution than the anion exchanger, it was used by Hollar *et al*. (1991) to separate the genetic variants A^1, A^2 and B of β-CN. Analyses were performed with 20 mM acetate buffer containing 6 M urea at pH 5. This method was used by Law (1993) to identify and measure the relative amounts of κ- and β-CN variants in milk samples from Friesian cows.

As illustrated in Fig. 3.9 (Humphrey and Newsome, 1984), anion exchange chromatography is a good method for separating the main whey proteins (Humphrey and Newsome, 1984; Andrews *et al*., 1985; Manji *et al*., 1985) with a resolution suitable for quantitative determinations

Improved chromatographic matrices with larger bead sizes, made the use of raw milk as a feed (at 37°C) possible for chromatographic separation or capture of dairy proteins. Fee and Chand (2006) purified Lf and lactoperoxidase in one step using cationic exchange SP Sepharose Big Beads™ (GE Healthcare).

(c) *Hydrophobic Interaction (HI) and Reversed-Phase (RP)-HPLC*

Although both techniques rely on hydrophobic interactions between a stationary phase and the solutes to be fractionated, their applications are quite different. With HI-HPLC, the solute is fixed in an aqueous solution at high ionic strength, and elution is achieved by lowering the ionic strength of the mobile phase. In RP-HPLC, fixation occurs in an aqueous solution of low ionic strength, and elution is obtained by increasing the hydrophobicity of the mobile phase. In HI-chromatography, the stationary phase is made of a polar material onto which non-polar groups (such as phenyl) are attached covalently, while the phases used in RP-HPLC are made of silica with non-polar group (such as C_5, C_8 or C_{18} alkyl chains) similarly attached. Unsubstituted residual silanol groups are subsequently "end-capped" by groups such as trimethylsilyl: Therefore, in theory, the stationary phases used in RP-HPLC can interact only with solutes through hydrophobic interactions.

RP-HPLC is now by far the most common type of chromatographic analysis used for the separation of whole casein on C_4, C_8 or C_{18} columns (Carles, 1986; Visser *et al*., 1986; Visser *et al*., 1991; Strange *et al*., 1991; Parris and Baginski, 1991) and whey proteins (Pearce, 1983; Humphrey, 1984; Parris and Baginski, 1991). This technique is well documented. Figure 3.10 shows the fractionation of the proteins in cheese whey on a C_6 column (Pearce, 1983). Improvements have been made subsequently in the direct analysis of skim milk by RP-HPLC (Visser *et al*., 1991; Bobe *et al*., 1998a; Bobe *et al*., 1998b). Urea, a reducing agent, and the addition of other components in the buffer, such as citrate (Visser *et al*., 1991), were required for the resolution

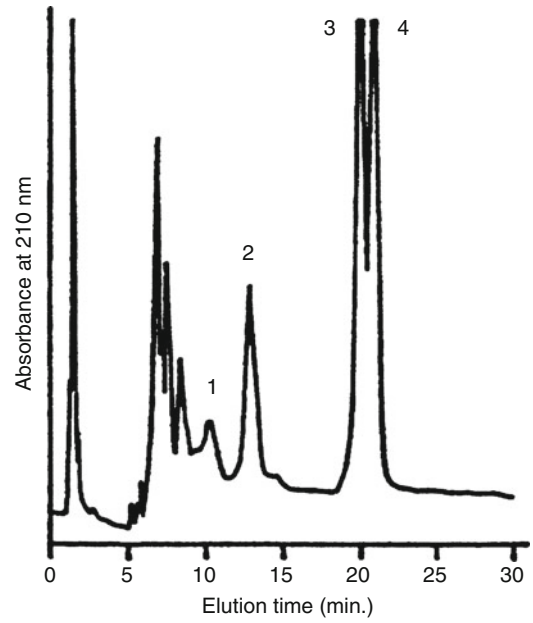

Fig. 3.10 RP-HPLC separation of defatted cheese whey on a Spherisorb C6 column (Pearce, 1983). Sample of whole, defatted cheese whey adjusted to pH 2.1; flow rate: 1 mL/min; solvent A: 0.15 M NaCl/HCl pH 2.1; solvent C: acetonitrile. Elution by multistage linear gradient from 0 to 48% B. *1* BSA, *2* α-La, *3* β-Lg B, *4* β-Lg A

of caseins and whey proteins. However, the presence of urea affects the separation of α-La and β-Lg (Visser *et al*., 1991) and reduces the β-CN peak relative to the peak areas of whey proteins, when the time interval between sample preparation and injection is increased (Groen *et al*., 1994). In addition, the co-elution of α-La with either α_{s1}-CN on a C_4 column (Parris and Purcell, 1990) or with β-CN B on a C_{18} column (Visser *et al*., 1991) limits the quantitative analysis of the major milk proteins. Bobe *et al*. 1998a, b) resolved these problems by using 6 M guanidine hydrochloride (GdnHCl), 0.37 mM sodium citrate and 19.5 mM DTT in the sample preparation (Bobe *et al*., (1998b). This method provides high resolution of the six major proteins in bovine milk, including their genetic variants, and makes quantitative analysis possible (Bobe *et al*., 1998a).

Quantification of native whey proteins, α-La and β-Lg in particular, is possible by using

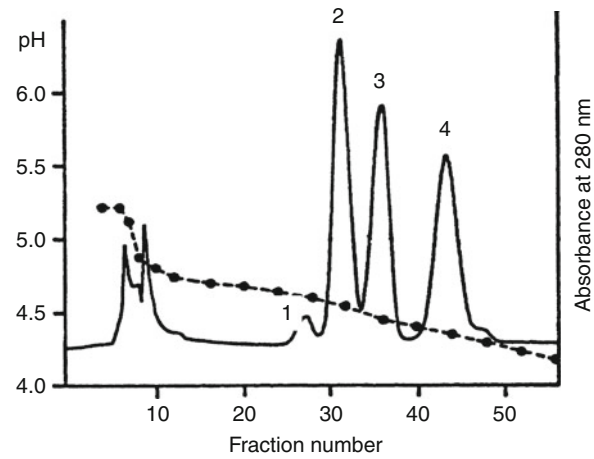

Fig. 3.11 Chromatofocusing separation of acid whey (Pearce and Shanley, 1981). Sample of milk serum concentrated and dialysed against starting buffer; Polybuffer 74 (diluted 1:10 in water and adjusted to pH 4.2) was run on the column before the sample was loaded. Elution with Polybuffer/HCl. Flow rate: 0.32 mL/min. *1* BSA, *2* β-Lg B, *3* β-Lg A, *4* α-La

C_4 or C_5 columns in acidic conditions, for example, 0.1% (w/w) trifluoroacetic acid. It was first noted by Beyer and Kessler (1989) that native and denatured/aggregated whey protein elutes differently under acidic conditions. The method has since been widely used for kinetic studies of whey protein denaturation (Croguennec *et al*., 2004; Kehoe *et al*., 2007a; Tolkach *et al*., 2005).

(d) *Other Chromatographic Techniques*

Due to its ability to separate proteins according to their phosphate content, chromatography on hydroxyapatite (HA) has been applied successfully to the separation of caseins (Kawasaki *et al*., 1986; Visser *et al*., 1986). However, the short viability of HA-HPLC columns and poor mechanical properties of the matrix used limit the development of such a technique. In spite of its high resolving power, chromatofocusing has seldom been applied to separate milk proteins (Pearce and Shanley, 1981; Fig. 3.11).

3.4.3 Mass Spectrometry

The mass spectrometer uses the difference in mass-to-charge (m/z) ratio of charged molecules (proteins, peptides) to separate them from each other in a mass analyser. It enables an exact determination of the molecular mass of a protein or peptide with very high sensitivity and resolution. The m/z measurements are made on molecular species in the gas phase. Protein and peptide analysis gained on the development of "soft" ionisation sources that allow the transfer of high molecular mass proteins or peptides from the condensed phase into the gas phase without destroying the molecules. A mass spectrometer is composed of three parts: an ionisation source that can transform proteins or peptides into ionised species in gas phase (ESI and MALDI), a mass analyser for the separation of the charged species according to m/z ratio (quadrupole (Q), time-of-flight (TOF), ion trap, ion cyclotron resonance and orbitrap mass analysers) and a detector that is usually an electrode onto which the ions falls (conversion of ion current to electrical current). Mass spectrometers are either one-stage instruments (MS) or complex multistage instruments (combinations of analysers in tandem (Q-q-Q, TOF-TOF) or hybrid (Q-TOF) configurations, MS/MS or MS^n) in order to combine the different capabilities of the mass analysers. Tandem and hybrid mass spectrometers are used to perform two or more sequential separations of ions by coupling two or more mass analysers and give the possibly of product ion scanning, precursor ion scanning or neutral loss scanning. MS/MS is routinely employed for primary amino acid sequencing and to determine the site and nature of a modification (e.g., post-translational, chemical). The mass spectrometer configuration determines the instrument performance (resolution, sensitivity,

mass accuracy, throughput, etc.), but no instrument offers all capabilities simultaneously. Each application needs a specific strategy for a mass spectrometer that is best suited for the analysis. Further details on the basics of mass spectrometry are available in El-Aneed *et al.* (2009). In this section, the ionisation sources (ES and MALDI) will be discussed in greater detail; the mass analysers coupled with the ionisation source will be only mentioned; the different types of detector will not be described. Several reviews on mass spectrometry applied to biological molecules (Arnott *et al*., 1993; Burlingame *et al*., 1998; Léonil *et al*., 2000; Domon and Aebersold, 2006; Mamone *et al*., 2009) and on milk proteins analysis (O'Donnell *et al*., 2004; Gagnaire *et al*., 2009) have been published. Emphasis here will be placed on the major contributions and the important analytical points of MS analysis applied to milk proteins.

(a) Mass Spectrometric Analysis

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) MALDI is a laser desorption mass spectrometry technique introduced by Karas and Hillenkamp (1988). Briefly, the sample is first mixed in solution with a large excess of a UV-absorbing matrix, typically a non-volatile low molecular weight aromatic acid (in case of a positive ionisation mode). Sinapinic acid (*trans*-3,5-dimethoxy-4-cinnamic acid), cinnamic acid and benzoic acid derivatives have often been chosen as matrices for milk proteins (Beavis and Chait, 1989). The mixture is placed on a MALDI plate and air-dried, resulting in a sample-matrix co-crystallisation, which is subsequently introduced on a target into the mass spectrometer source. With a short pulse (1–100 ns width) of a nitrogen laser beam, usually operating at 337 nm, the sample is energy-desorbed from the matrix into the gas phase and simultaneously ionised through a proton transfer from the matrix leading to vaporised ions. The amount of sample is usually in the low picomole range and a sample/matrix volume of about 1–2 μL with a large excess (about 100–50,000-fold molar excess) of matrix so that the laser beam will not hit the sample directly. Contrary to electrospray ionisation, this ionisation technique has the advantage of being tolerant to the presence of salts in the buffer and to other additives such as detergents (e.g., SDS). However, excessive amounts of salts can affect the signal. Because of the pulsed nature of MALDI, this technique is conveniently coupled to a time-of-flight (TOF) mass spectrometer, generally abbreviated as MALDI-TOF. The sample ions are accelerated in an electric field giving all ions the same kinetic energy and its mass-to-charge ratio is deduced from its flight time through a field-free drift of specified length and under vacuum; ions with higher m/z ratio move at lower velocity in the drift than ions with lower m/z ratio, then exhibit longer time of flight. MALDI usually produces singly charged ions, but higher charge states may also be observed. These latter may not be differentiated in the detection process but analysis of neighbouring peaks resulting from isotopic contribution may reveal the exact mass of the ionised molecules. Proteins with a molecular mass up to 300 kDa can be desorbed/ionised by MALDI. The time to accumulate a spectrum for a protein is based on the summation of the laser pulses (typically 10–100). Hence, the measurement of mass is made in a few seconds. The typical mass resolution (m/Δm) of MALDI-TOF improved considerably in recent years due to the development of a delayed extraction (Spengler and Cotter, 1990; Chaurand *et al*., 1999) and applying a reflectron before detection. Delay extraction narrows the initial kinetic energy distribution of ions with the same m/z ratio. The reflectron is an ion optic device that changes the path of the ions into the drift. Therefore, the time of flight of ions is increased, and ions with the same m/z but with different initial speed (due to slightly different kinetic energy) are focalised in the reflectron leading to a better resolution as well as more accurate mass measurements (El-Aneed *et al*., 2009). A drawback results in the loss of some ions in the reflectron reducing the sensitivity of the apparatus. Presently, the typical mass resolution of MALDI-TOF is in the range of 10,000–40,000 Da with an accuracy of 0.01% for linear TOF instruments.

Electrospray Ionisation Mass Spectrometry (ESI-MS). Electrospray ionisation is a process which involves passing a solution containing the

analyte molecules through a charged needle and then spraying it across a high potential difference (±3–5 kV). At the end of the needle, the solution disperses into a mist of small, highly charged droplets which contain the molecules (i.e., nebulisation). The charged droplets are desolvated either by gas flowing in the opposite direction to the spray (countercurrent gas, usually nitrogen) or by passing through a heated capillary. Solvent evaporation induces a large increase of the coulombic forces on the surface of the shrinking droplet. When these forces exceed the surface tension of the solvent, a coulomb explosion occurs resulting in the release of ionised molecules into the gas phase.

In the electrospray ionisation process, molecules have to be soluble in a preferably polar solvent. A key feature of the electrospray process is the formation of multicharged molecular species from analytes that are related to the charged sites carried by this molecule. Milk proteins usually give intense signals both in the negative-ion and positive-ion modes due to their high content in acidic (aspartic and glutamic acids, phosphoric acid groups) and basic (lysine, arginine, histidine) amino acid residues. Hence, milk proteins are usually detected with a relatively low m/z ratio. Proteins are positively or negatively charged depending on the pH of the diluting solvent. By the electrospraying process, proteins generate a series of peaks at $(MW + n \times m_H)/n$, MW being the relative molecular mass of the protein, m_H the molecular mass of a proton and n the number of charges. Consequently, the electrospray mass spectrum of a protein exhibits a coherent series of mass-to-charge peaks from which the molecular mass of the protein may be calculated very accurately using a computer algorithm for deconvoluting the ion series. Because this type of ionisation occurs at atmospheric pressure, this source has been coupled successfully with quadrupole and time-of-flight mass spectrometers. Typical resolution by quadrupole analyser is 1,000 (measured as $m/\Delta m$). A mass accuracy of 0.005% is obtained at the picomole level.

In the mass spectrometer, the sample is either infused directly into the source by infusion pumping or loaded onto a high-performance liquid column. Because electrospray ionisation requires a constant delivery of liquid, one of its major advantages is that it can be coupled easily to a liquid-based separation system such as HPLC or capillary zone electrophoresis (CZE). These configurations involving the coupling of a separation system and mass spectrometry are typically called LC-MS and CZE-MS, respectively. The coupling of chromatography to ESI-MS has the added advantage of purifying and concentrating the sample, allowing the analysis of protein mixtures. The development of nanospray technology, which operates at flow rate in the order of few nL/min, opens ESI-MS to the analysis of limited biological samples.

(b) Use of ESI-MS and MALDI-MS in the Analysis of Milk Proteins and Dairy Products. Milk is characterised by a great heterogeneity in its protein composition with few high-abundance proteins having numerous post-translational modifications (e.g., phosphorylation, glycosylation) and many low-abundance proteins and peptides. Beyond variations in composition, the milk is subjected to modifications during processing (e.g., enzymatic, chemical reactions) and storage, affecting further its nutritional, technological and sensory properties. In addition, buffalo, ovine or caprine milks are sometimes subjected to adulteration by bovine milk or by milk from different geographical origin in the case of production of products with protected designations of origin, because of limited amount available in some periods of the year or for economic reasons. Because of its ability to deliver high sensitivity characterisation of milk and dairy products, MS and MS/MS techniques gained a leading role for tracking low-abundance proteins, post-translational and process-induced modifications as well as fraudulent practice in dairy industry.

The determination of the molecular mass of proteins in milk using MS started in the 1990s (Marsilio *et al*., 1995; Léonil *et al*., 1995). Molecular mass values for the major proteins and several minor forms from skim milk using online LC-ESI-MS are presented in Table 3.6. The accuracy of the mass was in the 0.01% range, and

Table 3.6 Molecular mass determination of major proteins from skim milk by one-line LC-ESI-MS (from Léonil *et al.*, 1995)

Proteins	Molecular mass		
	Calculated[b]	Observed	Swaisgood (1992)
κ-CN A-1P	19,037.3	19,038 ± 3	19,038
[a]κ-CN A-2P		19,120 ± 5	–
κ-CN B-1P	19,005.5	19,006 ± 2	19,006
[a]κ-CN A-2P		19,087[c]	–
α_{s2}-CN A-11P	25,228.4	25,230 ± 2	25,238
[a]α_{s2}-CN A-13P		25,391[c]	25,400
α_{s2}-CN A-12P		25,311[c]	25,319
[a]α_{s2}-CN A-10P		25,151[c]	25,157
α_{s1}-CN B-8P	23,614.8	23,618 ± 2	23,623
[a]α_{s1}-CN B-7P		23,518	–
[a]α_{s1}-CN B-9P		23,698	–
[a]β-CN B-5P	24,092.4	24,093 ± 3	24,097
β-CN A^1-5P	24,023.3	24,024 ± 2	24,028
β-CN A^2-5P	23,983.3	23,985 ± 3	23,988
β-Lg B	18,278.3	18,278 ± 2	–
β-Lg A	18,363.4	18,365 ± 2	–

[a]Minor forms
[b]Calculated according to the values of average mass of amino acid residues reported by Feng *et al.* (1991)
[c]MW was measured from reconstructed spectrum, and standard cannot be calculated

the values measured are in close agreement with the theoretical values calculated from the published amino acid sequences.

Differences found between the measured MW of intact proteins and those calculated from the sequence may reveal the presence of either a mutation or a post-translational process. ESI-MS or MALDI-MS analyses allowed the detection of numerous genetic variants, particularly "silent" variants resulting from alternative splicing; they are responsible of the complexity of milk proteins, especially ovine and caprine α_{s1}-CN. These variants include β-CN F (Visser *et al.*, 1995), β-CN G (Dong and Ng Kwai Hang, 1998) and ovine α_{s1}-CN (Ferranti *et al.*, 1995; Mamone *et al.*, 2003), β-CN (Chianese *et al.*, 1995), caprine α_{s1}-CN (Roncada *et al.*, 2002) and β-CN (Neveu *et al.*, 2002).

Another application for MS analysis has been the location of an amino acid mutation in the primary sequence of the protein and on the identification of site and nature of post-translational modifications, such as phosphorylation and glycosylation. Location of these modifications is possible through protein sequencing. To do so, the isolated protein is subjected to a proteolytic digestion with specific enzymes such as trypsin, chymotrypsin, lysyl endopeptidase or *Staphylococcus aureus* V8 protease, to form a set of peptides which can be sequenced by mass spectrometry.

Protein sequencing by mass spectrometry is currently performed using tandem mass spectrometry (ESI-MS/MS or MALDI-MS/MS) or post-source decay (PSD)-MALDI-MS. Tandem mass spectrometry involves two consecutive stages of molecular mass analysis. The first stage selects the precursor ion of the target molecule (typically a peptide) subsequently fragmented by a process known as collision-induced dissociation (CID), in order to form fragment ions. The latter are transmitted into the second mass analyser where they are separated and detected. By this procedure (product ion scanning), a product ion spectrum is obtained along with the identification of the peptide's sequence (for reviews, see Smith *et al.*, 1990; Dongré *et al.*, 1997). (PSD)-MALDI-MS demonstrated also high potential for

protein sequencing (Spengler, 1997). Although the MALDI technique is considered a "soft" ionisation mode, metastable decomposition (random cleavage of peptide bonds, neutral molecule losses such as water or ammonia) occurs in the field-free drift of the TOF resulting in fragment ions. As the fragmentation occurs outside the acceleration field of the ion source, precursors and fragment ions move with the same velocity. They are separated according to m/z value in the reflectron. Protein sequencing by mass spectrometry allows the discrimination of all amino acids except Leu and Ile.

This approach was used by Visser *et al*. (1995) to identify the β-CN F 5P, which differs from β-CN A^1 by a replacement of a Pro residue by Leu at position 152; by Dong and Ng Kwai Hang, (1998) to identify β-CN G 5P by the same substitution at position 137 and by Neveu *et al*. (2002) to identify caprine β-CN C that differs in one substitution of residue Ala at position 177 by Val, from variant A. A similar approach was used for the identification and localisation of the carbohydrate chains and phosphorylated residues of caseins (Mollé and Léonil, 1995; Mamone *et al*., 2003; Holland *et al*., 2004). The different phosphorylated forms for the same family of molecules were easily identified by MS/MS by tracking a mass of 79 Da (PO_3^-) on the second MS analyser (precursor ion scanning) (Léonil *et al*., 2000).

The primary structure of the six most abundant bovine milk proteins was resolved in the early 1970s (see Eigel *et al*., 1984). For the other less abundant proteins and peptides, which principally come from somatic cells, leakage from the blood or mammary epithelia, the continuous developments in methodologies and instrument capabilities allowed the identification of new proteins and peptides (Fong *et al*., 2007; Smolenski *et al*., 2007; Fong *et al*., 2008; Reinhardt and Lippolis, 2008), some of which are located in the milk fat globule membrane and in different fractions of mature milk or colostrum (O'Donnell *et al*., 2004; Gagnaire *et al*., 2009).

Mass spectrometry may be used to determine the structural modifications of milk proteins during technological processing. A rapid evaluation of the effects of heat treatment on milk products is possible by performing a "fingerprinting" of the different proteins contained in the milk samples (Catinella *et al*., 1996a). A mass increment of 324 Da, referring to lactosylation, is readily detected by mass spectrometry analysis (Léonil *et al*., 1997). The extent of protein and peptide lactosylation depends on the thermal procedures used during industrial milk processing (Marvin *et al*., 2002; Meltretter *et al*., 2009; Arena *et al*., 2010). Arena *et al.* (2010) identified up to 271 non-redundant modification sites in 33 milk proteins for different milk samples (pasteurised, UHT and powdered milk for infant nutrition). A significant amount of lactosylated β-Lg was also quantified in commercial whey proteins products (Holt *et al*., 1999). Protein lactosylation (β-Lg and α-La) continues during the storage of UHT-treated milk (Holland *et al*., 2011). The application of ESI-MS and MALDI-MS to characterise the nature and extent of glycation of milk proteins has been reviewed by Oliver (2011). Heat treatments also induce whey protein denaturation and aggregation into mainly disulphide-linked aggregates. Several strategies were developed for the identification of disulphide bond reshuffling during heat treatment. One approach consists of an enzymatic hydrolysis of the heat-treated sample followed by the analysis of the mixture of peptides by LC-MS before and after reduction of the sample (Surroca *et al*., 2002; Livney *et al*., 2003). Another possibility results on the separation of the disulphide-linked peptides by diagonal electrophoresis prior to MS analysis. Diagonal electrophoresis means the separation of a mixture of peptides on a 2D electrophoresis using identical conditions for both dimensions. After migration in the first dimension, the peptides are exposed to performic acid in order to oxidise all cysteine residues into cysteic acid. After the second dimension, all the peptides except cysteine-containing peptides are located on the diagonal of the electrophoresis gel, because their migration is similar in both directions. The main drawback of the approach results on disulphide reshuffling during sample treatments (Visschers and de Jongh, 2005). However, reshuffling can be minimised if hydrolysis is conducted under low

pH condition (Swaisgood, 2005) or if the free thiol groups are previously blocked by a thiol-blocking agent (Kehoe *et al*., 2007b). From a tryptic digest, LC-MS(/MS) reveals that the first thiol/disulphide exchange reaction occurring during heat-induced unfolding of β-lg involves the free thiol group of Cys_{121} residue and the disulphide-bonded residues (Cys_{106}-Cys_{119}) leading to non-native monomers characterised by an exposed cysteine residue at position 119 and a non-native disulphide bond (Cys_{106}-Cys_{121}) (Croguennec *et al*., 2003). Similar work was carried out on commercial caseinates, which enabled the identification of two dephosphorylated forms of β-CN formed during processing procedures (Ward and Bastian, 1998). Holland *et al*. (2011) identified non-disulphide bond cross-linking between α_{s1}-CN and β-CN, as well as the deamination of Asn residue at position 129 of α_{S1}-CN during the storage of UHT-treated milks. Lysinoalanine cross-links were identified by LC-ESI-MS in calcium caseinate and milk powder, as well as dairy products fortified with such ingredients (Calabrese *et al*., 2009).

As far as milk products are concerned, the ability of mass spectrometry to identify peptides resulting from proteolysis has been demonstrated in numerous works (Ferranti *et al*., 1997; McSweeney and Fox, 1997; Alli *et al*., 1998; Gagnaire *et al*., 1999). Proteolysis occurs during either the ripening of cheese or the storage of milk products. For the former, mass spectrometry analysis allows great advances in the understanding of the ripening process and was used for the identification of bioactive peptides (Quiros *et al*., 2006; Quiros *et al*., 2007; Hayes *et al*., 2007) or antimicrobial peptides (Lopez-Exposito *et al*., 2006; Losito *et al*., 2007) released by milk coagulant, endogenous and microbial enzymes in such complex matrix. For the latter, Meltretter *et al*. (2008) identify using MALDI-MS the release of a peptide from the N-terminus of α_{S1}-CN in UHT-treated milk stored at room temperature.

Finally, as mass spectrometry analysis allows high sensitivity mapping of milks from several breeds of cow (Catinella *et al*., 1996b) and different species, it is considered a powerful tool to identify fraudulent practices in dairy industry (Liland *et al*., 2009; Mamone *et al*., 2009; Czerwenka *et al*., 2010; Nicolaou *et al*., 2011), for example, for the detection of the adulteration of buffalo milk and mozzarella by cows' milk using ESI-MS. Nicolaou *et al*. (2011) demonstrated by a combination of the whole MALDI-MS mass spectra and multivariate analysis the possibility to achieve accurate prediction of the level of milk species adulteration.

3.4.4 Secondary and Tertiary Structures

The secondary structure of proteins gives rise to a certain number of periodic structures such as α-helices, β-sheets and β-turns, as well as loops and random coils. These structures can be evaluated using spectroscopic methods such as circular dichroism (CD) and infrared (IR) spectroscopy. The higher levels of molecular organisation into tertiary and quaternary structures are studied using methods such as X-ray crystallography and nuclear magnetic resonance (NMR). As opposed to IR and CD, both methods provide sequence-specific information. The relationships between protein structure and function (biological and techno-functional) are of fundamental importance for understanding the way in which the molecule interacts with its surroundings. Given that each level of organisation is stabilised by different types of interactions, ionic bonds and hydrogen bonds, van der Waals and hydrophobic interactions, all of which are influenced by the physicochemical conditions, it is necessary to have techniques, such as FTIR, CD and NMR, to follow the conformational changes.

The detailed physical principle of these techniques has been described extensively in the literature. Thus, only a short description will be given in this section where emphasis will be placed on the recent methodological advances and their references.

(a) Infrared Spectroscopy

FTIR has been applied extensively to structural studies on milk proteins in order to establish the relationship between the structure and functionality of these proteins. A major advantage of

FTIR is that the samples can be examined readily in various forms: aqueous solutions, hydrated films, homogeneous dispersions or solids. Subirade *et al*. (1998) studied the effect of dynamic high pressure on the secondary conformation of thermally treated β-Lg. Changes in the microenvironment of amide C=O and N–H groups produced by perturbations such as denaturation may also be probed by FTIR spectroscopy. Parris and Purcell (1990) examined the thermal denaturation of whey proteins in milk by FTIR. Kumosinski and Farrell (1993) described a rapid quantitative procedure for determination of the global secondary structure of β-Lg by using H_2O rather than D_2O. This procedure provides a way to analyse both the amide I and II bands, which contrasts with H_2O where the amide II band is eliminated. Under these conditions, it was shown that the global structure of β-Lg was in good agreement with results obtained through X-ray diffraction analyses. Lefèvre and Subirade (1999) used FTIR to show the mode of interaction in β-Lg monomer-dimer equilibrium at pH 7 via intermolecular β-sheets, which corresponded well to X-ray results. Curley *et al*. (1998) used the same method to study the effect of calcium and temperature on the secondary structure of bovine casein. FTIR spectroscopy has been shown to detect and distinguish two different types of α-helical conformation in α-La, which constitutes a great advantage over CD spectrometry (Prestrelski *et al*., 1991). Improvements in the design of FTIR accessories have made it possible routinely to measure protein spectra in water. This was achieved by proper insulation and thermal control of transmission or ATR (attenuated total reflectance) cells, whereby the dominating and overlapping absorption band of water can be accurately subtracted from the protein spectra.

(b) Circular Dichroism Spectroscopy

Circular dichroism (CD) measures the difference in absorption between the two rotations (right and left) of circularly polarised light by an asymmetric molecule. This technique is non-destructive and is applied to molecules in solution. However, the CD signal is 10^{-3}–10^{-5} times the normal absorbance. A CD signal arises only where absorption of radiations by a molecule occurs, and thus spectral bands are readily assigned to specific structural feature of the molecules. Because of the presence of asymmetry in protein structure, proteins unequally absorb left- and right-circularly polarised light. The polarisation of the light leaving the optically active sample is elliptical. Ellipticity is the CD unit and is defined as the tangent of the ratio of the minor to the major elliptical axis. Ellipticity can be positive or negative, depending on whether the absorption coefficient for the left-circularly polarised component has values that are higher or lower than those for the right-circularly polarised component. CD spectroscopy measures the wavelength dependence of ellipticity along the absorbance spectrum of a molecule. CD signals from proteins arise principally from peptide bonds and aromatic residues in the far-UV (in the 170–240 nm range) and near-UV (in the 260–320 nm range) regions, respectively.

The peptide bonds of proteins and peptides give strong CD spectra for which two electronic transitions have been characterised: The π π* transition that occurs as a positive band around 190 nm and a negative band around 210 nm and the n π* transition registered as a negative band around 220 nm. The n π* transition of peptide bonds is sensitive to hydrogen bond formation and the secondary structures of protein in which are the peptide bonds. Hence, peptide bonds in α-helix, β-sheets or β-turns give very characteristic CD spectra in the far UV (Adler *et al*., 1973). The determination of protein secondary structures is computationally assisted using algorithms fitting the spectrum of the protein with the combination of the characteristic absorption of α-helix, β-sheets and β-turns structures. The different methods for sample preparation for CD measurements, as well as the methods for extracting the protein conformation from CD data, have been reviewed (Greenfield, 1996; Kelly *et al*., 2005). CD spectroscopy was reported to be more reliable for the quantification of helical motifs than for β-sheet, as well as unordered structures. It is important to note that estimation of the secondary structure in proteins requires spectral standard data established from CD spectra of reference proteins containing known amounts of

secondary structure (often determined by X-ray crystallography) (for references, see Greenfield, 1996; Kelly *et al*., 2005).

The contribution of aromatic residue to the near-UV spectra of the proteins constitutes a sensitive probe to identify change in protein conformation or ligand binding. Although aromatic side chains may be symmetric groups they are often thrust into an asymmetric environment leading to strong optical activity in CD analysis. CD analysis in the near-UV region gives evidence of the existence of "molten globule" structure in a protein characterised by a very weak near-UV CD spectra, reflecting the high mobility of aromatic side chains (Kelly *et al*., 2005).

Application to Milk Proteins. CD spectroscopy has been applied extensively to the structural characterisation of β- (Andrews *et al*., 1979), κ- (Loucheux Lefebvre *et al*., 1978; Raap *et al*., 1983), α_{s1}- (Haga *et al*., 1983) and α_{S2}- (Hoagland *et al*., 2001) caseins. A predominant percentage of β-sheet conformation was found in all caseins. CD analysis of β-CN indicates the presence of approximately 32% of β-sheet, 28% turns, 21% unordered and 20% helix (Farrell Jr *et al*., 2001). Although these CD data are appropriate for globular proteins, such as β-Lg and α-La, the assignment of a secondary structure can be misleading when applied to proteins having an open conformation, such as caseins (Sawyer and Holt, 1993). CD analysis is often used to identify change in the conformation of whey proteins due to technological processing.

(c) X-Ray Crystallography

Principle. X-ray crystallography remains the technique of choice for determining the precise three-dimensional atomic structure of proteins. For X-ray analysis, the crystal structure is necessary. Accordingly, only proteins that can be crystallised are amenable to X-ray crystallography. Based on observations of the crystal structure obtained at high resolution, it is now known that 10% or more of polypeptide chain residues adopt multiple conformations (Smith *et al*., 1986). Consequently, it appears that the conformation determined by X-ray crystallography is less "static" than inferred so far.

The wavelength range of X-rays corresponds to the size of the diffracting structure (atomic radii and lattice constant). When an X-ray beam bombards a crystal, the electrons surrounding the nucleus of each atom either bend or diffract it to give a specific pattern known as X-ray diffraction. This X-ray diffraction pattern is related to the 3-dimensional electron density distribution within the molecule. Although all the electrons in the molecule participate in each diffracted beam, their contribution varies according to phase considerations. The accurate determination of phases (known as the phase problem) requires that a suitable heavy-atom derivative be incorporated without distorting the crystal. From this, the electron density map may be obtained from the diffraction pattern from which a three-dimensional structure model is built, aided by computers for mathematically interpreting this pattern. An important step is the validation of the final model (i.e., the fitting of the model to the experimental data). Significant advances in crystallisation methods in conjunction with new methods for determining phase, such as multiple anomalous dispersions (MAD), and the advent of bright synchrotron radiation sources make it possible to obtain results routinely that are accurate to 0.1 and 0.2 Å (with a resolution in the 1.5–2 Å range) (Carter and Jr. and Sweet, R.M, 1997).

Applications to Milk Proteins. X-ray crystallographic methods have been used successfully to determine the three-dimensional structure of bovine β-Lg with a 1.8 Å resolution (Brownlow *et al*., 1997) and α-La with a 1.8 Å resolution (Warme *et al*., 1974). Caseins cannot be crystallised. The three-dimensional models have been proposed based on energy-minimised algorithms.

(d) Nuclear Magnetic Resonance (NMR)

Principle. NMR is increasingly becoming an important tool for full structural determination of proteins due to the development of multidimensional NMR. As often underlined in the literature, it is a technique that is both theoretically and technically complex. The physical principles will be described briefly here and excellent descriptions can be found in specialised reviews. This

section highlights recent advances in NMR for which references will be cited therein.

NMR can determine the structure of proteins in the 15–30 kDa range, with a resolution comparable to 2.5 Å resolution crystal structures (Garrett *et al*., 1997). A great advantage of NMR over X-ray crystallography is that the structure determination is performed in an aqueous solution as opposed to in a crystal lattice. Accordingly, NMR is appropriate to study the dynamic structure of molecules such as it occurs in a solution (Wüthrich, 1989). The main limitation of this technique is the necessity to have a high protein concentration in a highly purified form, typically about 1 mM for a sample volume in the 0.3–0.5 mL range. The four most abundant elements (H, C, N and O) in proteins have an overall spin property (non-zero spin) due to the occurrence of natural isotopes with non-zero spin, making them observable in a NMR experiment. The precession of such nuclei generates a magnetic moment that may be oriented either with or against an external magnetic field. This orientation may be reversed if a quantum of the correct energy is absorbed. The resulting nucleus immediately relaxes back to its ground state by emitting a quantum. The rate (frequency) of precession for each isotope is dependent of the strength of the external field and is unique for each isotope. The Fourier transformation of the signal yields the conventional one-dimensional NMR spectrum in which the intensity of the emitted energy is given as a function of the frequency of the quanta. Such spectra provide information on the environment of the atoms. Further structural information arises from the introduction of 2D-NMR which allows the collection of data on magnetisation transfer between pairs of protons both through bond (J coupling) and through space (the nuclear Overhauser effect, NOE). In other words, J coupling between pairs of protons separated by three or fewer covalent bonds can be measured, as well as NOE arising from dipole-dipole coupling between two protons through space usually separated by a distance less than 5 Å. Thus, the NOE constitutes a sensitive probe for the close proximity in space, and this technique is called NOESY.

A 2D-NMR spectrum as a diagonal and cross-peaks symmetrically placed on either side of the diagonal indicate the existence of an interaction between two spins. Several 2D-NMR techniques can be used, depending on the investigated interactions. In a correlation COSY (COSY, homonuclear shift correlation spectrometry) experiment, the cross-peaks are due to through-bond scalar correlation, while in a NOESY experiment, they arise from through-space correlation (Clore and Gronenborn, 1998). It was shown that the secondary structure of a protein can be determined by NMR from the knowledge of the chemical shifts of the amide and α-CH proton (Wishart *et al*., 1992). However, the complete assignment of individual resonance for a protein needs the combination of COSY and NOESY spectra. Once the backbone is assigned, the assignment of the side-chain proton is performed by another 2D spectroscopic technique, total correlation spectroscopy (TOCSY) in addition to COSY. In TOCSY, the cross-peaks arise from all of the connectivity within the spin system and are not limited to those arising from three bonds.

The general strategies used to resolve the 2D-NMR spectra have been described by Wüthrich (1989) and comprehensive reviews on 2D and higher-dimensional NMR developments have been published by Oschkinat *et al*. (1988) and Clore and Gronenborn (1991, 1998).

Diffusion NMR has also been used for analysing proteins in solution. In principle, an increasing magnetic pulse-field gradient is applied to the sample and monitoring the rate of decay as a function of magnetic field strength can be used to determine the diffusion coefficient of the protein. Diffusion is related to particle size and through manipulation of the Stokes-Einstein equation it is possible to determine the diffusion coefficient, that is, the rate of decay of the molecule in solution, of a protein. This can then be used to calculate accurately the hydrodynamic radius (RH) of the protein using an internal standard of 1, 4-dioxane as a reference. The diffusion coefficient is a reliable guide to the apparent molecular weight

and tertiary structure of a protein, for example, the RH of the protein increases when the protein unfolds. The diffusion coefficient is determined from the slope of the integrated the peak area of the aromatic region (6.5–8.5 ppm) versus the pulse-field intensity (Jones *et al*., 1997).

Application to Milk Proteins. NMR has been applied to all major milk proteins. 1D-NMR on isolated or whole caseins previously gave unresolved spectra which were difficult to interpret. Investigations have been performed on β-Lg and α-La. 2D-NMR has been used to characterise denatured and unfolded states of β-Lg (Ragona *et al*., 1997). NMR analysis was performed under acidic conditions to prevent molecular aggregation that hampered the spectrum (Molinari *et al*., 1996). Alexandrescu *et al*. (1993) studied the binding of Ca by α-La using NMR techniques.

NMR was used to probe the perpetually molten globule form of α-La (named All-Ala, where the cysteines have been replaced by alanine) and compared to the structure of the molten globule form induced by pH reduction (Redfield *et al*., 1999). NMR was also used to determine the structure of the molten globule form of α-La at pH 7, both with urea-denaturation and the use of All-Ala-α-La (Rösner and Redfield, 2009).

3.5 Immunochemical Methods

3.5.1 Introduction

Over the last decades, the quality of dairy products has improved significantly. Producers have been encouraged to produce milk of high hygienic and compositional quality. Thus, the dairy industry has a constant need to develop and use rapid and reliable analytical tests to identify the raw materials and technologies applicable to dairy products.

Immunochemistry has often been used to detect admixtures (in the case of fraud), contaminants (like melamine), pathogens, antibiotics, viruses, etc. This chapter will only focus on the immunoassays developed for quantifying proteins (caseins, whey proteins, enzymes…) in milk and dairy products. Proteins have conformations that vary according to their amino acid sequence, the number of disulphide bridges and the biophysical environment. Thus, when a given protein (antigen) is injected into an animal, the host recognises it as a foreign substance and develops an immune response towards it. This response is characterised by the production of antigen-specific molecules, called antibodies. When placed together, antigens and their corresponding antibodies bind specifically, even in a mixture.

The serum obtained after immunising an animal with an antigen is called an antiserum. It is a serum that contains antibodies of different specificity and therefore is also called "polyclonal antibody." The latter have the ability to bind with antigens via different sites, called epitopes or antigenic determinants. Köhler and Milstein (1975) developed a procedure to raise a unique population of antibodies directed against one epitope. These "monoclonal antibodies" have since been used widely in the development of immunoassays because they are extremely specific reagents.

3.5.2 General Characteristics of the Immunochemical Techniques

One of the most important characteristics of immunochemical techniques is their high specificity. These techniques allow for the detection and/or quantification of a single molecule, even in a complex protein mixture. Furthermore, by using very specific antibodies, it is possible to discriminate different forms of the same molecule; thus, it has been demonstrated that an antibody can be specific for the heat-denatured form of a protein, but not for its native form (Negroni *et al*., 1998; Jeanson *et al*., 1999).

Sensitivity is also a major characteristic of immunochemical techniques. In milk, they can quantify molecules at very low concentrations, such as a few ng/mL. It is even possible to increase immunoassay sensitivity by using fluorescence or luminescence for the revelation of the reaction.

Finally, immunochemical techniques can provide results rapidly, if optimised. For instance,

quantification of a protein using a biosensor can be completed within a few minutes.

3.5.3 Description of the Immunochemical Techniques Applied to Dairy Products

(a) *Enzyme-Linked Immunosorbent Assay (ELISA)*

ELISA was first described by Engvall and Perlmann (1971). At that time, ELISAs were performed in test tubes. Later, Scherrer and Bernard (1977) performed this technique in a microplate.

An ELISA consists of a two-pronged strategy: (1) the reaction between the immunoreactants (antibody with the corresponding antigen) and (2) the detection of that reaction using an enzyme, bound to the reactants, as an indicator. This technique is based on the fact that, at alkaline or neutral pH, a protein can be immobilised by non-covalent binding onto a solid phase, such as the polystyrene of a microplate. This technique offers several advantages, which are listed in Table 3.7.

Table 3.7 Advantages of enzyme immunoassays (from Tijssen, 1988)

Very high sensitivity, detectability and specificity are possible
Equipment required is relatively cheap
Assays may be very rapid and simple
Reproducibility is high and evaluation is objective
Feasible under field conditions
No radiation hazards
Reagents are relatively cheap and generally of long shelf-life
Versatility of assays may be increased significantly by the great variety and specific properties of enzymes
Full advantage of the properties of monoclonal antibodies may be achieved with ELISAs

The different types of ELISAs are represented in Fig. 3.12. Direct and indirect ELISAs are non-competitive methods which consist of coating the antigen onto the microplate. This step may induce conformational changes in the antigen, resulting in the modification of antibody binding. Therefore, direct and indirect ELISAs are often used for qualitative tests. Another non-competitive method is the sandwich ELISA. Here, the antibodies are immobilised to trap antigens from crude extracts. This technique requires that the antigens have at least two epitopes.

Competitive methods include competitive and inhibition ELISAs. Competitive ELISAs are based on the competition of enzyme-labelled antigen with the antigen present in the test sample

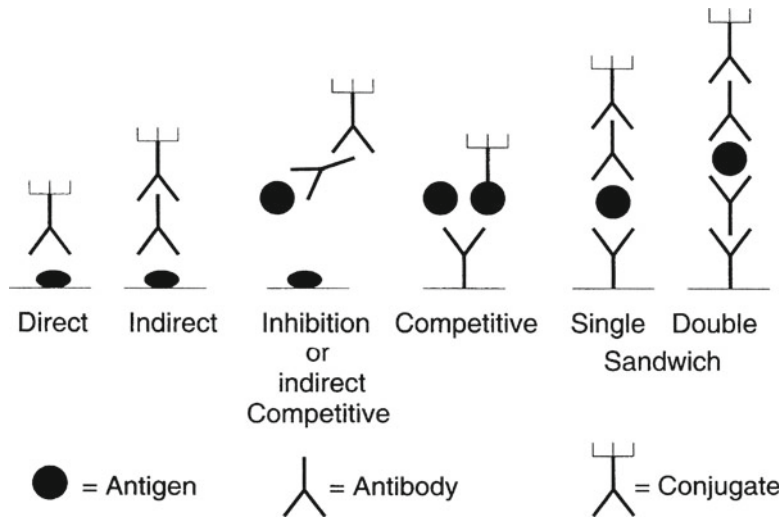

Fig. 3.12 The different types of ELISA

for the antibody on the solid phase. Inhibition (also called competitive-indirect) ELISAs are based on the inhibition of the reaction between the enzyme-labelled antibodies and the immobilised antigen by free antigen present in the test or calibration sample. The amount of enzyme immobilised on the solid phase is inversely proportional to the amount of free antigen present in the incubation mixture.

Quantitative assays are mostly inhibition, competitive or sandwich ELISAs, where the antigen-antibody immune complex is made in solution and keeps the antigen in its native form.

There has been some misunderstanding on the definition of ELISA sensitivity. The limit of detection or detectability of an ELISA corresponds to the lowest concentration which gives a signal that is significantly different from that of the background values (= ability to detect). In contrast, sensitivity is defined by the dose–response curve: It corresponds to the change in response (dR) per unit amount of reactant (dC) and equals dR/dC, the slope of the titration curve. Thus, an ELISA can have a high detection limit and, at the same time, be extremely sensitive.

In the same way, there is sometimes confusion between the terms "accuracy" and "precision." Accuracy is the conformity of a result to an accepted standard value or true value. Precision, however, is defined as the degree of agreement between replicate measurements of the same quantity and may be of very low accuracy.

(b) *Precipitation in Gel*

These techniques are based on the diffusion of an antigen and/or an antibody in a gel. A precipitate forms as the antigen and the antibody interact. Some of these techniques are quantitative. Radial immunodiffusion (Mancini *et al*., 1965) has been the most widely used quantitative technique. This technique involves the diffusion of an antigen (antibody) through an agarose gel which contains the corresponding specific antibody (antigen). This leads to the formation of circles, proportional to the antigen concentration. Radial immunodiffusion is simple, easy and rapid to perform even if 1 day is needed to obtain results. The most important drawbacks, however, are its lack of sensitivity (limit of detection of few μg/mL) and the need to precipitate antigens and antibodies when complexed.

(c) *Immunoblotting*

Immunoblotting involves the separation of the different constituents of a mixture by electrophoresis, their transfer onto a membrane and their visualisation using an antibody. It can be a sensitive technique, especially when luminescence is used for protein visualisation (10 pg of a protein can be detected in this case). However, this technique can hardly be quantitative and can take a day to complete.

(d) *Microparticle-Enhanced Nephelometric Immunoassay*

The microparticle-enhanced nephelometric immunoassay is based on the ability of antigen-coated microspheres to agglutinate in the presence of corresponding antibodies. The agglutination builds large microsphere clusters and induces turbidity, scattering the light of an incident monochromatic beam. The scattered light is measured with a specifically designed nephelometer.

This technique is extremely simple to perform and has been proven reliable. However, it has shown several disadvantages:

- An underestimation of the results in excess of antigen
- A lack of sensitivity (limit of detection, around 1 μg/mL)
- The use of clear media in order to avoid interference of medium turbidity with the formation of immune complexes
- The use of "plurivalent" antigens for the constitution of the antigen-antibody network.

(e) *Biosensors*

A biosensor can be defined as a device that combines a biological recognition mechanism with a transducer, which generates a measurable signal in response to changes in the concentration of a given biomolecule. One component of the interaction to be studied is covalently immobilised to the matrix, and other interactants are passed over the sensor in solution. The mass change at the sensor surface, reflecting the progress of the interaction studied, is monitored in real time. The technique, which does not require molecular labels for detection, can measure mass changes down to 10 pg/mm^2. Biosensor

technology has been used in food analysis since the mid-1990s. During recent years, SPR-based biosensors have been applied to milk proteins for quantifying caseins in milk (Muller-Renaud *et al*., 2003, 2004, 2005), following casein-casein (Marchesseau *et al*., 2002; Thompson *et al*., 2010) or casein-polysaccharide interactions (Thompson *et al*., 2010). More recently, Dupont *et al*. (2011) examined the potential of SPR-based immunosensors used as probes for exploring the surface of the casein micelle.

(f) *Antibody Arrays*

An antibody microarray is a specific form of protein microarray. A collection of capture antibodies is spotted and fixed on a solid surface, such as glass, plastic or silicon chip, for the purpose of detecting antigens. An antibody microarray is often used for detecting protein expressions from cell lysates in general research and special biomarkers from serum or urine for diagnosis applications. The great advantage of this technology is to allow a high-throughput simultaneous analysis of thousands of antigen-antibody interactions. Applications related to milk and dairy products are limited and involve the diagnosis of milk-related pathologies like allergy (Gaudin *et al*., 2008) or the detection of minor constituents of major biological interest such as cytokines in colostrum (Kverka *et al*., 2007). More recently, antibody arrays have also been applied to monitor the hydrolysis of caseins during dairy product digestion using collection of monoclonal antibodies of known specificity (Dupont *et al*., 2010a).

3.5.4 Application of the Immunochemical Techniques to Dairy Product Analysis

(a) *Quantitative Determination of Proteins in Milk and Milk Products*

Caseins. Few quantitative techniques have been developed in recent years for estimating the concentrations of the different caseins in dairy products. This is probably because in most dairy products, caseins are susceptible to hydrolysis over time (during cheese ripening or milk storage). Thus, immunoassays based on the production of an antibody directed against a specific CN will also bind to the peptides originating from the parent CN and will thus be biased.

Several microparticle-enhanced nephelometric immunoassays have been developed for the quantification of α_s- and κ- (Collard Bovy *et al*., 1991; Humbert *et al*., 1991; Montagne *et al*., 1995) and β-CNs in milk (Montagne *et al*., 1995). Montagne *et al*. (1995) measured the α-, β- and κ-CNs in 1300 milk samples collected from 50 herds over a period of 13 months. Their results confirmed the well-established relations between composition parameters; evolution and variability of the CNs; influence of breed, season, calving and feeding; and consequences for cheesemaking properties. More recently, Muller-Renaud *et al*. (2003, 2004, 2005) proposed a strategy in order to quantify only intact caseins in milk and not their degradation products. This idea was to develop sandwich immunoassays with antibodies specific to the N- or C-terminal extremities of the casein. An immunosensor was developed allowing the simultaneous quantification of α_{s1}-, β- and κ-casein in raw and heat-treated milk (Dupont and Muller-Renaud, 2006).

Finally, a SPR-based biosensor was used to study the topography of the casein micelle. Forty-four monoclonal antibodies specific from different epitopes of the four caseins (Johansson *et al*., 2009) were captured onto the sensor surface through a covalently immobilised anti-mouse IgG. Then, casein micelles or EDTA-solubilised micelles were injected and the interactions with the monoclonal antibodies were monitored in real time. Epitopes accessible at the periphery of the micelle were identified and corresponded to the C-terminal extremity of the κ-casein but also to hydrophobic domains of α_{s1}-, α_{s2}- and β-caseins (Dupont *et al*., 2011).

Whey Proteins. Whey proteins have been studied widely using immunochemical techniques because they:

- Have different sensitivities to heat-denaturation and they can be used as markers of heat-treatment
- Are involved in allergies to dairy products
- Can constitute a model for globular conformation, as is the case for β-Lg

Furthermore, many techniques have been developed for their quantification in dairy products.

β-*Lactoglobulin*. An ELISA has been developed for β-Lg evaluation in dairy products (Haque and Pruett, 1993). However, this test can differentiate between the genetic variants A and B. In human milk, bovine β-Lg has also been quantified by ELISA (Makinen Kiljunen and Palosuo, 1992; Fukushima *et al*., 1997) and by radioimmunoassay (Kilshaw and Cant, 1984). The presence of bovine β-Lg at low concentrations (6–45 μg/L vs 3–4 g/L in bovine milk) in human milk is probably due to its diffusion into the milk of lactating woman who have consumed bovine milk. Therefore, β-Lg can cause an allergic response even in breast-fed infants.

α-*Lactalbumin*. Duranti *et al*. (1991) and Jeanson *et al*. (1999) developed ELISAs to quantify α-La in raw and heat-treated commercial milks. Marchal *et al*. (1991) determined α-La concentration in milk using a microparticle-enhanced nephelometric immunoassay. More recently, Dupont *et al*. (2004) developed an immunosensor for quantifying simultaneously the native and heat-denatured forms of α-La. This technique allowed a sharp characterisation of the intensity of the heat treatment to which it had been subjected without disposing of the original raw milk (Feinberg *et al*., 2006). Finally, Indyk (2009) used the same technology to develop a rapid assay for quantifying α-La in consumer milk, colostrum, whey protein concentrates and infant formulae, the temporal change during early bovine lactation and a preliminary study of thermal denaturation.

Immunoglobulins. Clinical studies have shown the important potential role of using milk Ig's to prevent mortality in infants exposed to infectious diseases (Narayanan *et al*., 1983). Oral administration of hyperimmune milk derived from cows immunised with non-viable pathogens has been shown to protect against intestinal bacterial infections (Kobayashi *et al*., 1991). Other studies have shown the effectiveness of bovine milk IgGs from non-immunised cows in preventing gastrointestinal diseases in infants (Ballabriga, 1982). Thus, the use of milk from either immunised or non-immunised cows as a source of IgGs requires sensitive, simple, time-efficient and relatively inexpensive methods for routine screening of milk IgGs. Immunological methods developed for the detection of Ig in milk include single radial immunodiffusion (Levieux, 1991), immunoelectrophoresis (Al Mashikhi and Nakai, 1987), immuno-nephelometry (Montagne *et al*., 1991) and ELISA (Kummer *et al*., 1992).The influence of standards and antibodies in immunoassays to quantify Ig's in milk has been shown by Li Chan and Kummer (1997). Losso *et al*. (1993) developed a solid-phase particle concentration fluorescence immunoassay based on the agglutination of antibody, covalently bound to carboxyl-polystyrene particles, with the antigen, which is in turn detected by a fluorescent-labelled antibody by epifluorometry. This assay is simple to perform, sensitive (5 ng/mL), accurate, repeatable and rapid (less than 1 h). Finally, a SPR-based biosensor assay was developed for the determination of IgG in bovine colostrum and milk (Indyk and Filonzi, 2003).

Bovine Serum Albumin. Levieux and Ollier (1999), using SRID, studied the changes in concentrations of the IgG, β-Lg, α-La and BSA in milk throughout the first 16 milkings *postpartum*. The concentrations decreased abruptly (IgG, β-Lg, BSA) or slowly (α-La) from the first milking to the last. These results were tabulated to calculate the excess whey proteins that would be obtained if colostrum or early milk was added illegally to the milk supply. BSA in milk has also been determined by ELISAs to establish the effect of milking frequency on its concentration (Stelwagen and Lacy Hulbert, 1996). It was observed that once-daily milking increased the level of BSA. However, during subsequent twice-daily milking, BSA level remained high.

Lactoferrin. Competitive ELISAs have been developed to quantity lactoferrin in bovine milk using rabbit polyclonal antibodies (Le Magnen *et al*., 1989) and specific monoclonal antibodies (Shinmoto *et al*., 1997). A sensitive sandwich ELISA allowed the quantification of lactoferrin

in cheese. It showed that the concentration of this protein depended on the cheese variety with quite large amounts in Swiss-type cheese (Dupont *et al*., 2006). The same assay was also applied for the quantification of lactoferrin in goat milk (Lefier *et al*., 2010). Finally, Campanella *et al*. (2009) developed immunosensors for the quantification of lactoferrin and IgG in milk from different species (cow, goat, buffalo).

Enzymes. More than 60 different enzymes are reported in milk. From a technological viewpoint, proteases are probably the most important. Three major types of proteases, each of different origins, can be distinguished in dairy products:

- Indigenous enzymes, such as plasmin and cathepsin D, which are naturally present in milk. Plasmin has been shown to play a major role during the ripening of hard cheese (Grappin *et al*., 1985), whereas the role of cathepsin D has not been determined fully.
- Milk-clotting enzymes (chymosin, pepsin, microbial or plant proteinases), which are used to coagulate milk, are also essential for the ripening of cheeses, particularly low-cook cheeses.
- Bacterial proteinases and peptidases, for example, from starter and non-starter microorganisms, or from *Pseudomonas*.

Because of their specificity (in secondary proteolysis in cheese, quantification of an enzyme in a protein-rich medium, possibility to distinguish an enzyme from its inactive precursor) and their sensitivity (enzymes are usually present at very low concentrations in milk or cheese), immunoassays are suitable tools for the study of enzymes in dairy products.

Plasmin. Dupont *et al*. (1997) developed an ELISA for differential titration of plasmin (PLM) and its precursor plasminogen (PLG) in milk using two monoclonal antibodies, one PLG specific and the other cross-reacting equally with PLM and PLG. Using this test, they observed a dramatic increase in the concentrations of PLM and PLG in milk at the end of lactation (Dupont *et al*., 1998). Then, these authors applied the ELISA to cheese and observed high concentrations of PLM in hard cheeses due to the activation of PLG to PLM during cooking (Dupont and Grappin, 1998). A microparticle-enhanced nephelometric assay was also developed for bovine PLG in milk using a polyclonal rabbit antiserum directed against both PLG and PLM (Marchal *et al*., 1995).

Cathepsin D. Cathepsin D in milk was demonstrated using immunoblotting by Larsen and Petersen (1995). According to Larsen *et al*. (1996), cathepsin D is present mainly in the whey where its concentration, evaluated by competitive ELISA, is around 0.3 μg/mL.

Coagulant Enzymes. ELISAs have also been developed to quantify residual chymosin in dairy products (Andersson *et al*., 1989; Boudjellab *et al*., 1994). In high-cooked hard cheese, chymosin is partially denatured by the cooking temperature used during cheesemaking, limiting its role in proteolysis in this type of cheese. Sensitive ELISAs have also been proposed for the quantification of *Rhizomucor miehei* proteinase (Rauch *et al*., 1989) and porcine pepsin (Boudjellab *et al*., 1998) in cheese.

Bacterial Peptidases. ELISAs for the detection and the quantification of *Lactococcus* peptidases in cheese have been developed (Chapot Chartier *et al*., 1994; Laan *et al*., 1996), as well as an immunoblotting technique to visualise the release of peptidases from *Lactobacillus* on lysis (Valence *et al*., 1999).

Proteases of Psychrotrophic Bacteria. Although ultrahigh temperature treatment applied to beverage milk destroys contaminating microorganisms, proteases from psychrotrophic bacteria and particularly from *Pseudomonas fluorescens* are resistant to this treatment and may cause proteolysis and destabilisation of the milk. Thus, ELISAs were developed to detect proteases from *Pseudomonas fluorescens* in milk (Clements *et al*., 1990). However, this approach has some limitations because the protease produced can vary immunologically from one psychrotrophic strain to the next (Birkeland *et al*., 1985). Another way for assessing this type of proteolytic activity is to quantify the products released by these heat-resistant enzymes from the caseins. Finally, a third strategy was followed by Dupont *et al*. (2007) who designed a

monoclonal antibody specific for the peptide bond Phe_{105}-Met_{106} of κ-casein, the major cleavage site of *Pseudomonas fluorescens* proteases, by immunising mice with a synthetic peptide covering this part of the sequence. This antibody interacted with the cleavage site as long as it was intact but did not when the peptide bond hydrolysed. The inhibition caused by the monoclonal antibody specific for Phe_{105}-Met_{106} permitted detection of UHT milks in the process of being destabilised. However, the major drawback of this approach is that when destabilisation is evidenced, there is nothing to do besides removing the products from the market.

(b) *Structural and Conformational Modifications of Milk Proteins*

Chemical or physical treatments, such as enzymatic proteolysis or heat denaturation, may lead to changes in the structure and/or conformation of milk proteins. These modifications can be studied easily using immunochemical techniques. In fact, when an epitope is hydrolysed by an enzyme, it will not be recognised by a specific antibody. In the same way, protein denaturation may induce conformational modifications, degradation or even aggregation of proteins that will result in changes in their recognition by specific antibodies. Thus, antibodies can be efficient probes for characterising conformational structures involved in the biology of dairy proteins (e.g., interaction with ligands, hypersensitivity reactions) and for studying conformational changes that occur upon physical treatment (e.g., denaturation).

Denaturation of Milk Proteins. Several studies have been performed to determine the heat treatment undergone by milk using immunochemical techniques. Duranti *et al*. (1991) developed an ELISA for quantification of α-La in milk. Using this technique, they showed that the "immunodetectable" α-La in heat-treated milk samples decreased with the severity of the heat treatment. Jeanson *et al*. (1999) developed two inhibition ELISAs for quantification of the native and heat-denatured forms of α-La in milk using specific monoclonal antibodies. Combination of these two techniques, together with expression of the result as a percentage of denatured α-La, allowed the assessment of the degree of heat treatment undergone by a milk without the need to analyse its original raw milk. However, due to the resistance of α-La to heat denaturation, this technique was more suitable for the study of high-pasteurised, UHT and sterilised milk than minimum pasteurised or thermised milk. Comparable work has been done by Negroni *et al*. (1998) on β-Lg. Levieux (1980) used radial immunodiffusion to study the denaturation of α-La and β-Lg in milk and whey. This author observed that both molecules were more resistant to heat denaturation in whey. Indeed, denaturation in serum leads to conformational modifications of the proteins that are reversible on cooling. In milk, however, denaturation of these two proteins by heat treatment causes the aggregation between α-La and β-Lg and the binding of the complex onto κ-CN (Elfagm and Wheelock, 1978). This aggregation prevents "renaturation" of the protein.

Proteolysis in Milk. One approach to study the hydrolysis of milk proteins is to detect and quantify the enzymes responsible for this phenomenon. The other way is to study directly the products produced by an enzyme. For instance, to study the release of the caseino-macropeptide (CMP) from κ-CN by proteinases of *Pseudomonas*, Picard *et al*. (1994) and Prin *et al*. (1996) developed a quantitative ELISA and a microparticle-enhanced nephelometric assay, respectively. However, both used a rabbit polyclonal anti-κ-CN antiserum. Thus, CMP and κ-CN had to be separated by ultrafiltration. Using this technique, Prin *et al*. (1996) observed that the concentration of CMP measured in milk ultrafiltrates was underestimated by about 25%. Other authors studied the microorganism responsible for this proteolysis. Immunoassays to directly detect *Pseudomonas fluorescens* strains and related psychrotrophic bacteria in milk were developed (Gonzalez *et al*., 1993, 1994, 1997). Unfortunately, the antibodies raised against a protein from the cell envelope of *Ps. fluorescens* were strain specific and bound more weakly the other strains of *Ps. fluorescens* (Gonzalez *et al*., 1993). Using polyclonal (Gonzalez *et al*., 1994) or monoclonal (Gutierrez *et al*., 1997) antibodies raised against live cells, these authors slightly improved the sensitivity of the technique.

In Cheese. Proteolysis in cheese has been studied by immunoblotting using antibodies specific for the different caseins and for different fragments of the caseins released by enzymatic activity. Addeo *et al*. (1995a) used polyclonal antibodies specific for β- and α_s-CNs to study proteolysis during ripening of six European hard cheeses. Pizzano *et al*. (1997) raised polyclonal antibodies specific for α_{s1}-CN by immunising rabbits with bovine α_{s1}-CN (f141–148) and α_{s1}-CN (f139–149). They used the polyclonal anti- α_{s1}-CN (f139–149) to determine proteolysis of α_{s1}-CN in Parmigiano Reggiano cheese samples throughout 180 days of ripening (Pizzano *et al*., 1998).

In the Human Gut. After ingestion, milk and dairy products will be extensively hydrolysed in the gut during digestion. This proteolytic process is currently being studied extensively in order to determine the nature of the bioactive peptides released during the gastrointestinal tract and evaluate their potential action on human health, but also see if processing conditions were modifying the pattern of peptides released. It has been shown that heat treatment tended to increase casein resistance to digestion by causing the formation of thermally induced casein-whey protein aggregates (Dupont *et al*., 2010a, b). This could partly explain why caseins, known as being very sensitive to hydrolysis due to their flexible structure, could partly resist digestion and generate allergic reactions in the newborn.

(c) *Adulteration of Dairy Products*

Immunoassays have been used widely to detect adulteration of milk and dairy products (see reviews by Moatsou and Anifantakis, 2003; Pizzano *et al*., 2011). One of the major adulterations of dairy products involves fraudulent substitution of ewes' milk by less-expensive cows' milk. This substitution can become a serious problem in cheese manufacture as the origin of the milk influences the sensory characteristics of the final product. Thus, for economic as well as for ethical reasons, there has been a need for analytical procedures that can detect the addition of cows' milk to ewes' or goats' milk to protect ovine and caprine milk products from adulteration and to assure the consumers of product quality.

It is possible to produce a species-specific antibody that will detect only a cow marker in ewes' milk. Three different strategies can be used to obtain species-specific antibodies:

- A polyclonal antibody can be raised against a protein and adsorbed against the protein of the other species. The protocol for rendering a polyclonal serum specific for an antigen has been described by Avrameas and Ternynck (1969).
- When the sequence of the protein from different species is known, it is possible to select a species-specific peptide, to synthesise it and to use it to immunise rabbits. The resulting polyclonal serum will recognise only the targeted marker. All the procedures for animal immunisation with synthetic peptides have been described by Tam (1994).
- Monoclonal antibodies raised against a protein can be directed against species-specific sequences and thus prove valuable in detecting adulterations of dairy products.

Cow/Ewe and Cow/Goat. Numerous immunological techniques have been developed during the last decade for the detection of fraudulent addition of cows' milk to ewes' milk. Bitri *et al*. (1993) raised a polyclonal serum against a κ-CN peptide, that is, κ-CN (f139–152), and developed a competitive ELISA for the detection of 0.25% of cows' milk into ewes' milk. The antibody produced was directed against the heat-resistant CMP. Therefore, detection of heat-treated bovine milk was possible. A lower limit of detection (0.01%) was obtained by Levieux and Venien (1994), who used a bovine-specific monoclonal antibody against β-Lg. β-CN (Anguita *et al*., 1995, 1996, 1997) and γ_3-CN (Richter *et al*., 1997) have also been used as bovine-specific antigens to detect addition of cows' milk to ewes' milk. It has been emphasised that the use of antibodies against casein allows the detection of added heat-treated milk, whereas antibodies directed against heat-labile WPs do not.

When milk proteins have undergone hydrolysis, for example, during cheese ripening, other immunological techniques should be applied. Addeo *et al*. (1995b), using polyclonal antibodies against bovine γ_2 or β-CNs, and an immunoblotting procedure, were able to detect the adulteration

Table 3.8 Detection of milk and cheese adulteration using immunochemical techniques

References	Product	Adulteration	Marker	Antibody	Technique	Limit of detection (%)
Bitri *et al.* (1993)	Milk, cheese	Cow/goat	κ-CN f 139–152	Polyclonal	Competitive ELISA	0.25
Levieux and Venien (1994)	Milk	Cow/ewe Cow/goat	β-Lg	Monoclonal	ELISA	0.01
Anguita *et al.* (1995)	Milk	Cow/ewe Cow/goat	β-CN	Monoclonal	Indirect ELISA	?
Addeo *et al.* (1995b)	Cheese	Cow/ewe Cow/buffalo	β-CN	Polyclonal	Immunoblotting	0.5
Haza *et al.* (1996)	Milk	Goat/ewe	α_{s2}-CN	Monoclonal	Indirect ELISA	0.5
Anguita *et al.* (1996)	Milk, cheese	Cow/ewe	β-CN	Monoclonal	Immunostick ELISA	1 (Milk)
Beer *et al.* (1996)	Cheese	Cow/ewe Cow/goat	β-1 g	Adsorbed Polyclonal	Inhibition ELISA	0.5 (Cheese)
Molina *et al.* (1996)	Cheese	Cow/goat Cow/ewe	β-1 g	Polyclonal	Immunoblotting	0.1–0.2
Haza *et al.* (1997)	Milk	Ewe/goat	α_{s2}-CN	Monoclonal	Indirect ELISA Inhibition ELISA	0.5 0.25
Anguita *et al.* (1997)	Milk, cheese	Cow/ewe Cow/goat	β-CN	Monoclonal	Competitive ELISA	0.5
Hiesberger and Brandl (1997)	Milk-curd	Soy/milk	(Commercial)	(Commercial)	Inhibition ELISA	?
Richter *et al.* (1997)	Milk, cheese	Cow/goat Cow/ewe	γ_3-CN	Polyclonal	Inhibition ELISA	0.1
Hurley *et al.* (2006)	Milk, cheese	Cow/goat Cow/sheep Cow/buffalo	IgG	Monoclonal Polyclonal	Sandwich ELISA	0.01 0.001 0.001
Costa *et al.* (2008)	Milk, cheese	Cow/ewe Goat/ewe	(Commercial)	(Commercial)	ELISA	0.2
Addeo *et al.* (2009)	Milk, cheese	Cow/buffalo	γ2-CN	Anti-peptide antibody	Immunoblotting	0.25

of ewes' milk cheese with bovine milk constituents at a level of 5% and 0.5%, respectively. Beer *et al*. (1996), using a bovine-specific polyclonal serum against β-Lg and an indirect competitive ELISA, and Molina *et al*. (1996), using a monoclonal antibody against β-Lg and an immunoblotting procedure, detected the adulteration of ewes' cheese by cows' milk. More recently, Anguita *et al*. (1997) and Richter *et al*. (1997) used bovine-specific β-CN monoclonal antibodies and bovine-specific γ_3-CN polyclonal serum, respectively, as a tool to detect adulteration (by ELISAs).

Most of the techniques used to detect cows' milk in ewes' products milk were also applied successfully to detect cows' milk in goats' milk dairy products (Table 3.8).

Goat/Ewe. ELISA techniques have also been developed for the detection of goats' milk in ewes' milk (Haza *et al*., 1996) and for ewes' milk in goats' milk (Haza *et al*., 1997) using monoclonal antibodies against α_{s2}-CN.

Soy Proteins/Milk Proteins. Another type of adulteration of dairy products involves the addition of soy milk into bovine milk. Thus, Hewedy and Smith (1990) have developed an ELISA that allowed the detection of soy proteins in bovine milk, whereas Hiesberger and Brandl (1997) used a commercially available ELISA, developed for the detection of soy protein in meat products, to detect soy proteins in bovine milk and cheese.

3.5.5 Significance and Possible Developments in Immunochemical Methods

Although most of the immunoassays used by the dairy industry have been developed to ensure the safety of dairy products (detection of pathogens, quantification of toxins, drug residues, pesticides, etc.), immunochemical techniques have also been used widely in milk protein analysis. They provide a major analytical tool for estimating constituents that otherwise would be impossible to detect using the traditional methods of chemical analysis. Indeed, because of their high sensitivity, they are more useful for the detection and quantification of molecules present at low concentrations but that are nonetheless of significant technological or physiological importance. They can also be performed very quickly and with a minimum amount of sample preparation. Because of their high specificity, immunochemical techniques are particularly well adapted for monitoring conformational modifications of proteins due to denaturation or proteolysis and for detecting sequence differences in related proteins. In the near future, more and more specific probes, such as monoclonal antibodies, will be needed. Novel forms of detection, such as chemiluminescence and cascade-amplified systems, will improve significantly the already high sensitivity of the immunoassays. It is also possible that the strong need for "on-farm" control will result in the development of end user-friendly immunoassays. It is likely that the trend will be towards different solid-phase configurations, such as "dipsticks" or latex bead assays. Also, biosensors based on immunological principles that have the advantage of providing continuous "on-line" measurements and are therefore better suited to process control than current immunoassays will undergo a strong development.

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Chemistry of the Caseins

4

T. Huppertz

4.1 Introduction

Milk protein constitutes an important part of the human diet. For the neonate, milk or infant formula is the only type of food consumed; however, whereas milk does not constitute a major part of the diet after the neonatal stage of most other mammals, the human diet in many parts of the world continues to include high levels of dairy products. The popularity of milk proteins in the human diet is undoubtedly a result of the combination of their excellent nutritional value and high level of functionality. The relative ease of isolation of proteins from milk has led not only to the creation of a wide variety of functional and nutritional milk protein ingredients, but also to milk proteins being the best characterized of all food proteins. The primary structures of all milk proteins have been determined and for all the major whey proteins, three-dimensional structures have been elucidated. Because of the fact that attempts to crystallize caseins have thus far remained unsuccessful, the full secondary and tertiary structure of the caseins remains to be elucidated. Although caseins have higher flexibility than typical globular proteins, e.g. whey proteins, the previous classification of caseins as random coil or natively denatured proteins appears inaccurate as a definite degree secondary and tertiary structure has been identified for the caseins.

The aim of this chapter is to provide the current state-of-the-art with respect to the chemistry of caseins. As with previous reviews on this topic by Swaisgood (1982, 1992, 2003), casein composition and nomenclature, chemical composition and primary structure of the caseins, post-translational modification, secondary structures as well as physicochemical properties of caseins, such as self-association and the interactions with calcium, will be covered. For studies on the isolation of casein, the reader is referred to Swaisgood (2003). Higher order structures of caseins and the structure and stability of the association colloids in which caseins naturally exist, i.e. casein micelles, are outside the scope of this chapter and are covered in Chaps. 5 and 6, respectively. The focus in this chapter will be on the caseins in bovine milk; interspecies variability in casein composition is covered in Chap. 13.

4.2 Casein Composition and Nomenclature

The American Dairy Science Association Committee on the Nomenclature, Classification and Methodology of Milk Proteins originally defined the bovine caseins as those phosphoproteins that precipitate from raw milk by

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Table 4.1 Current and former nomenclature of caseins and major peptides derived therefrom. Reference proteins are printed in italics

Current	Former
α_{s1}-CN	
α_{s1}-CN A-8P	α_{s1}-CN A
α_{s1}-CN B-8P	*α_{s1}-CN B*
α_{s1}-CN B-9P	α_{s0}-CN
α_{s1}-CN C-8P	α_{s1}-CN C
α_{s1}-CN D-9P	α_{s1}-CN D
α_{s1}-CN E-8P	α_{s1}-CN E
α_{s2}-CN	
α_{s2}-CN A-10P	α_{s6}-CN A
α_{s2}-CN A-11P	*α_{s4}-CN A*
α_{s2}-CN A-12P	α_{s3}-CN A
α_{s2}-CN A-13P	α_{s2}-CN A
β-CN	
β-CN A^1-5P	β-CN A^1
β-CN A^2-5P	*β-CN A^2*
β-CN A^3-5P	β-CN A^3
β-CN C-4P	β-CN C
β-CN D-4P	β-CN D
β-CN E-5P	β-CN E
κ-CN	
κ-CN A-1P	*κ-CN A*
κ-CN B-1P	κ-CN B

acidification to pH 4.6 at 20°C (Jenness *et al.*, 1956). Subsequent reports by the committee recommended that the caseins could be differentiated according to their relative electrophoretic mobility in alkaline polyacrylamide or starch gels containing urea, with or without β-mercaptoethanol (Whitney *et al.*, 1976) or, more recently, according to their primary amino acid sequences (Eigel *et al.*, 1984; Farrell Jr *et al.*, 2004). Accordingly, four gene products can be identified: α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN) and κ-casein (κ-CN). Typical concentrations of α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN in bovine milk are 12–15, 3–4, 9–11 and 2–4 $g\,L^{-1}$, respectively, and the caseins account for ~75–80% of total milk protein. For all caseins, various genetic variants have been identified. In addition, all caseins show considerable micro-heterogeneity, arising from post-translational modification; all caseins are phosphorylated, whereas glycosylation has been shown only for κ-CN. As discussed in further detail later, α_{s1}-CN, α_{s2}-CN and β-CN are classified as the calcium-sensitive caseins, whereas κ-CN is calcium insensitive. In addition to the aforementioned gene products, γ-caseins and λ-caseins have been identified, which arise from the hydrolysis of β-CN and α_{s1}-CN, respectively, by the indigenous milk proteinase, plasmin. Enzymatic hydrolysis of milk proteins by plasmin is outside the scope of this chapter and is dealt with in detail in Chap. 12.

Current nomenclature of caseins and some casein fractions, as well as former classifications by which they were known, is shown in Table 4.1. In such nomenclature, a Latin letter indicates the generic variant of the proteins, whereas differences in the degree of post-translational modification are indicated by an Arabic number, followed by the letter P to indicate that the post-translational variation arises from phosphorylation. For example, α_{s1}-CN B-8P refers to genetic variant B of α_{s1}-CN containing eight phosphorylated amino acid residues. For each of the caseins, one of the variants outlined in Table 4.1 is considered to be the reference protein; these reference proteins are α_{s1}-CN B-8P, α_{s2}-CN A-11P, β-CN A^2-5P and κ-CN A-1P.

4.3 α_{s1}-Casein

4.3.1 Primary Structure of α_{s1}-Casein

The α_{s1}-CN family represents ~40% of total casein in bovine milk. The reference protein for the α_{s1}-CN family is α_{s1}-CN B-8P, with ExPASy entry name and file number of CAS1_Bovin and P02662, respectively. The amino acid sequence of α_{s1}-CN B-8P, which predominates in the milk of *Bos taurus* and was first established by Mercier *et al.* (1971) and Grosclaude *et al.* (1973), is shown in Fig. 4.1. The protein consists of 199 amino acid residues, with 8 of the 16 Ser residues in the protein being phosphorylated, i.e. Ser_{45}, Ser_{47}, Ser_{64}, Ser_{66}, Ser_{67}, Ser_{68}, Ser_{75} and Ser_{115} (Mercier *et al.*, 1971). In α_{s1}-CN B-9P, previously denoted α_{s0}-CN, Ser_{41} is also phosphorylated

1							10								20
Arg-	Pro-	Lys-	His-	Pro-	Ile-	Lys-	His-	Gln-	Gly-	Leu-	Pro-	Gln-	Glu-	Val-	Leu-	Asn-	Glu-	Ans-	Leu-
21							30								40
Leu-	Arg-	Phe-	Phe-	Val-	Ala-	Pro-	Phe-	Pro-	Glu-	Val-	Phe-	Gly-	Lys-	Glu-	Lys-	Val-	Asn-	Glu-	Leu-
41							50								60
Ser-	Lys-	Asp-	Ile-	Gly-	SerP-	Glu-	SerP-	Thr-	Glu-	Asp-	Gln-	Ala-	Met-	Glu-	Asp-	Ile-	Lys-	Gln-	Met-
61							70								80
Glu-	Ala-	Glu-	SerP-	Ile-	SerP-	SerP-	SerP-	Glu-	Glu-	Ile-	Val-	Pro-	Asn-	SerP-	Val-	Glu-	Gln-	Lys-	His-
81							90								100
Ile-	Gln-	Lys-	Glu-	Asp-	Val-	Pro-	Ser-	Glu-	Arg-	Tyr-	Leu-	Gly-	Tyr-	Leu-	Glu-	Gln-	Leu-	Leu-	Arg-
101							110								120
Leu-	Lys-	Lys-	Tyr-	Lys-	Val-	Pro-	Gln-	Leu-	Glu-	Ile-	Val-	Pro-	Asn-	SerP-	Ala-	Glu-	Glu-	Arg-	Leu-
121							130								140
His-	Ser-	Met-	Lys-	Glu-	Gly-	Ile-	His-	Ala-	Gln-	Gln-	Lys-	Glu-	Pro-	Met-	Ile-	Gly-	Val-	Asn-	Gln-
141							150								160
Glu-	Leu-	Ala-	Tyr-	Phe-	Tyr-	Pro-	Glu-	Leu-	Phe-	Arg-	Gln-	Phe-	Tyr-	Gln-	Leu-	Asp-	Ala-	Tyr-	Pro-
161							170								180
Ser-	Gly-	Ala-	Trp-	Tyr-	Tyr-	Val-	Pro-	Leu-	Gly-	Thr-	Gln-	Tyr-	Thr-	Asp-	Ala-	Pro-	Ser-	Phe-	Ser-
181							190								200
Asp-	Ile-	Pro-	Asn-	Pro-	Ile-	Gly-	Ser-	Glu-	Asn-	Ser-	Glu-	Lys-	Thr-	Thr-	Met-	Pro-	Leu-	Trp	

Fig. 4.1 Amino acid sequence of bovine α_{s1}-CN B-8P

(Manson *et al.*, 1977). De Kruif and Holt (2003) identified two centres of phosphorylation in α_{s1}-CN, i.e. f41–51, containing Ser_{41} (only in the 9P variant), Ser_{45} and Ser_{47}, and f61-70, containing residues Ser_{64}, Ser_{66}, Ser_{67} and Ser_{68}. These centres of phosphorylation are crucial in the stabilization of the calcium phosphate nanoclusters in the casein micelles (De Kruif and Holt, 2003).

The amino acid composition and properties of α_{s1}-CN B-8P are shown in Table 4.2. Based on amino acid composition, the molecular mass of the protein prior to post-translational modification is estimated at ~23.0 kDa, which increases to ~23.6 kDa as a result of the phosphorylation of eight Ser residues. Based on the primary sequence, a pI of ~4.9 would be expected for α_{s1}-CN, but this decreases by ~0.5 pH units through the phosphorylation of the eight Ser residues. Such values are in line with reported pI of α_{s1}-CN varying from 4.4 to 4.8 (Trieu-Cuot and Gripon, 1981; Eigel *et al.*, 1984). The aliphatic index, grand average hydropathicity (GRAVY) and hydrophobicity all suggest a moderately hydrophobic protein. α_{s1}-CN B-8P contains 25 amino acid residues capable of carrying a positive charge and 40 capable of carrying a negative charge. A distribution of the charge over the polypeptide chain is shown in Fig. 4.2, which clearly highlights a positively charged N-terminus and a high concentration of negative charges, including the two clusters of phosphorylation, between residues 30 and 80. A moderate and even distribution of positive and negative charges is found between residues 81 and 150, whereas the remainder of the protein, with the exception of the 10 amino acid C-terminus, is largely unchanged. Distribution of hydrophobicity, according to the scale of Tanford (1962), of α_{s1}-CN B is also shown in Fig. 4.2. In this scale, positive values represent a hydrophobic character whereas negative values represent a hydrophilic character. Some distinct patches of significant hydrophobicity can be observed, i.e. residues 20–35 and 160–175.

Table 4.2 Amino acid composition and properties of α_{s1}-CN B-8P

Amino acid	α_{s1}-CN B-8P		
Ala	9	Total residues	199
Arg	6	Positively charged residues (Lys/Arg/His)	25
Asn	8	Negatively charged residues (Glu/Asp/SerP)	40
Asp	7	Aromatic residues (Tyr/Phe/Thr)	20
Cys	0		
Gln	14	Molecular mass	
Glu	25	Based on primary structure	22,975 Da
Gly	9	Including phosphorylation	23,599 Da
His	5		
Ile	11	pI	
Leu	17	Based on primary structure	4.91
Lys	14	Including phosphorylation	4.42
Met	5		
Phe	8	Extinction coefficient at 280 nm[a]	25900 M^{-1} cm^{-1}
Pro	17		
Ser	16	Absorbance at 1 g L^{-1} at 280 nm[a]	1.127
Thr	5		
Trp	2	Aliphatic index[a]	75.43
Tyr	10		
Val	11	Grand average of hydropathicity (GRAVY)[a]	−0.704
		$H\Phi_{ave}$ (kJ/residue)[a]	4.89

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

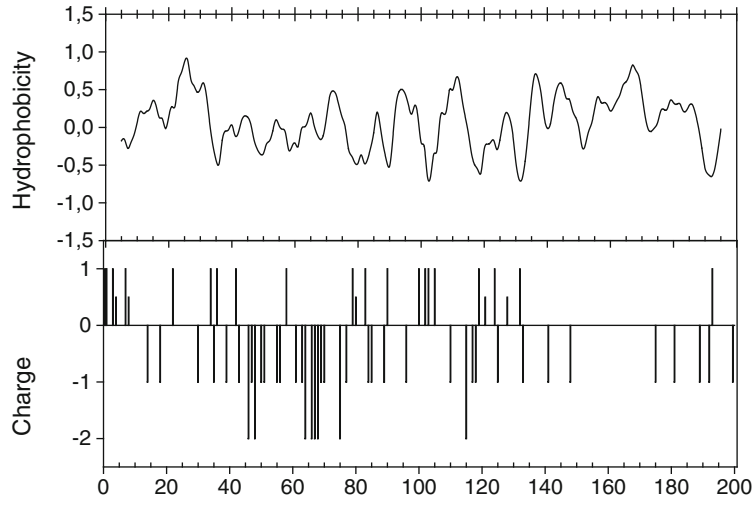

Fig. 4.2 Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of α_{s1}-CN B-8P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

4.3.2 Genetic Variation of α_{s1}-Casein

In addition to the B-variant of α_{s1}-CN, a number of other genetic variants have been identified, an overview of which is shown in Table 4.3. In α_{s1}-CN A, the amino acid residues 14–26 are missing as a result of exon skipping (Grosclaude *et al.*, 1970); this variant has been found in Holstein Friesians, Red Holsteins and German Red cattle (Ng-Kwai-Hang *et al.*, 1984; Grosclaude, 1988; Erhardt, 1993). Variant α_{s1}-CN C predominates in the milk of *Bos indicus* and *Bos grunniens* (Eigel *et al.*, 1984) and contains Gly instead of Glu at position 192 (Grosclaude *et al.*, 1969). In α_{s1}-CN D, which has been found in various breeds in France (Grosclaude, 1988) and Italy (Mariani and Russo, 1975) as well as in Jerseys in the Netherlands (Corradini, 1969), the Ala residue at position 53 is replaced by a phosphorylated Thr residue (Grosclaude *et al.*, 1972). A replacement at position 59 of Gln by Lys and at position 192 of Glu by Gly yields α_{s1}-CN E, which is found in *Bos grunniens* (Grosclaude *et al.*, 1976), whereas α_{s1}-CN F contains Leu instead of SerP at position 66 and is found in German Black and White cattle (Erhardt, 1993). Finally, α_{s1}-CN G was discovered in Italian Brown cows (Mariani *et al.*, 1995), but no amino acid sequence has been reported for this variant to date, whereas α_{s1}-CN H arises from an eight amino acid deletion at positions 51–58 (Mahe *et al.*, 1999).

Table 4.3 Differences in the amino acid sequence of genetic variants of αs1-casein compared to α_{s1}-CN B-8P

	Position					
Variant	14–26	51–58	53	59	66	192
A	Deleted					
B			*Ala*	*Gln*	*SerP*	*Glu*
C						Gly
D			ThrP			
E				Lys		Gly
F					Leu	
G						
H		Deleted				

4.3.3 Secondary Structure of α_{s1}-Casein

The secondary structure of α_{s1}-CN has been studied using a number of different approaches. While Fourier transform infrared (FTIR) spectroscopy studies by Byler and Susi (1986) found no secondary structure in α_{s1}-CN, other studies have reported varying degrees of secondary structure elements in α_{s1}-CN. The percentage of α-helix in α_{s1}-CN has been estimated as 5–15% (Herskovits, 1966), 8–13% (Byler *et al.*, 1988), 20% (Creamer *et al.*, 1981) or 13–15% (Malin *et al.*, 2005). For β-sheet, values of 17–20% were reported (Byler *et al.*, 1988; Creamer *et al.*, 1981), whereas Malin *et al.* (2005) reported 34–46% extended β-sheet-like structures in α_{s1}-CN. In addition, 29–35% β-turn structures have been reported for α_{s1}-CN (Byler *et al.*, 1988). In addition, the presence of polyproline II structures in α_{s1}-CN is evident from Raman optical activity spectra (Smyth *et al.*, 2001). Higher order structures of caseins are described in further detail in Chap. 5.

4.3.4 Self Association of α_{s1}-Casein

Self-association of α_{s1}-CN is characterized by progressive strongly pH- and ionic strength-dependent consecutive self-association to dimers, tetramers, hexamers, etc. (Ho and Waugh, 1965; Payens and Schmidt, 1965, 1966; Schmidt and van Markwijk, 1968; Swaisgood and Timasheff, 1968; Schmidt, 1970a, b). At pH 6.6 and ionic strength >0.003, the monomers exist in a rapidly equilibrating equilibrium with oligomers; increasing ionic strength results in increasing association constants and the appearance of larger oligomers (Ho and Waugh, 1965; Schmidt and van Markwijk, 1968; Schmidt, 1970b). The free energy for formation of the various oligomers is comparable; hence, all species exist at appreciable concentrations, but they occur to different extents. At an ionic strength of 0.003, only monomers are present, whereas at an ionic strength of 0.01, a monomer–dimer equilibrium exists; at an

ionic strength of 0.2, dimers and tetramers are favoured, while the formation of larger oligomers becomes progressively less favourable (Ho and Waugh, 1965; Schmidt, 1970b). Likewise, as the pH is increased above 6.6, the electrostatic repulsive free energy increases, resulting in smaller association constants yielding a lowered degree of association (Swaisgood and Timasheff, 1968). The larger association constants, and resulting much stronger association, of α_{s1}-CN C compared to α_{s1}-CN B can be explained by the change in electrostatic free energy (Schmidt, 1970a) due to its smaller net charge. However, α_{s1}-CN D behaves identically to α_{s1}-CN B (Schmidt, 1970a) although its net charge is greater than that of α_{s1}-CN B. It should be noted that the α_{s1}-CN B to α_{s1}-CN D substitution, at position 53 (Table 4.3) occurs in the polar domain, whereas the α_{s1}-CN B to α_{s1}-CN C substitution, at position 192 (Table 4.3), occurs in the hydrophobic domain which is more likely to be in the association contact surface. Enzymatic deimination of five of the six Arg residues of α_{s1}-CN reduces the susceptibility of the protein to self-association (Azuma *et al.*, 1991).

4.3.5 Interactions of α_{s1}-Casein with Calcium

When considering the interactions of α_{s1}-CN, or any of the other caseins, with calcium, or other cations, two aspects should be considered, i.e. the binding of calcium by the protein and the calcium-induced precipitation of the protein by calcium. α_{s1}-CN is one of the calcium-sensitive caseins; precipitation of α_{s1}-CN occurs in the range of 3–8 mM $CaCl_2$ (Schmidt, 1969; Bingham *et al.*, 1972; Toma and Nakai, 1973; Dalgleish and Parker, 1980; Aoki *et al.*, 1985; Farrell Jr *et al.*, 1988) and occurs more readily for α_{s1}-CN B than for α_{s1}-CN A (Farrell Jr *et al.*, 1988). When $CaCl_2$ concentration exceeds ~0.1 mM, the solubility of α_{s1}-CN increases again, due to the salting-in effect (Farrell Jr *et al.*, 1988). Calcium-induced precipitates of α_{s1}-CN are readily solubilized in 4 M urea, suggesting that no calcium-induced cross-linkage of proteins occurred and that the driving forces behind the calcium-induced association are driven by hydrogen bonding and hydrophobic interactions in the absence of electrostatic repulsion (Aoki *et al.*, 1985).

An extensive investigation into the calcium-binding and calcium-induced precipitation of α_{s1}-CN by Dalgleish and Parker (1980) highlighted that the binding of calcium by the protein decreases considerably with increasing ionic strength. In addition, the concentration of calcium required to induce precipitation of α_{s1}-CN also increases with increasing ionic strength, but not proportionally to calcium binding, i.e. the degree of calcium binding which is required to induce precipitation of α_{s1}-CN decreases with increasing ionic strength (Dalgleish and Parker, 1980). Calcium binding by α_{s1}-CN decreases when pH decreases below 7.0, but decreasing pH increases the concentration of calcium required to induce precipitation of α_{s1}-CN (Dalgleish and Parker, 1980). Calcium-induced aggregation of α_{s1}-CN was described as a monomer–octamer equilibrium, followed by Smoluchowski aggregation in which only the octamers participate (Dalgleish et al., 1981). Dephosphorylation reduces the number of calcium-binding sites on the protein and also reduces the stability of α_{s1}-CN to calcium-induced precipitation (Yamuachi *et al.*, 1967; Bingham *et al.*, 1972; Aoki *et al.*, 1985). Deimination of Arg residues in α_{s1}-CN enhances calcium binding, as well as the stability of the protein to calcium-induced precipitation (Azuma *et al.*, 1991).

Detailed analyses of the effects of calcium binding on α_{s1}-CN have indicated several equilibria. The addition of up to 1 mM $CaCl_2$ to α_{s1}-CN induces an exothermic process, possibly hydrogen-bond formation (Holt *et al.*, 1975), binding of calcium only to phosphorylated Ser residues (Ono *et al.*, 1976) and the transfer of Tyr and Trp residues from an aqueous to an apolar environment (Ono *et al.*, 1976). As the concentration of $CaCl_2$ is increased from 1 to 3 mM, the aforementioned exothermic phase is followed by an increasingly endothermic reaction, possibly hydrophobic interactions (Holt *et al.*, 1975); the burying of the aromatic chromophores is abated (Ono *et al.*, 1976), whereas calcium binding by both phosphorylated Ser and carboxylate-containing residues occurs (Ono *et al.*, 1976);

turbidity increases slightly to a plateau level (Holt *et al*., 1975) and increasing numbers of bent-chain polymers are observed (Dosaka *et al*., 1980). Finally, between 3 and 5 mM calcium chloride, the reaction becomes very endothermic (Holt *et al*., 1975); binding of calcium, primarily to carboxylate-containing residues, continues (Ono *et al*., 1976); the turbidity increases dramatically (Holt *et al*., 1975) and precipitation eventually occurs. These results suggest that binding of Ca^{2+} to high-affinity phosphoseryl clusters in the polar domain alters its interaction with the hydrophobic domain, bringing about a conformational change in that domain which allows some association to occur. Further binding to carboxyl residues throughout the structure reduces the electrostatic repulsion and, consequently, interaction of the hydrophobic domains leads to the formation of large aggregates.

4.4 α_{s2}-Casein

4.4.1 Primary Structure of α_{s2}-casein

The α_{s2}-CN family constitutes up to 10% of the total casein fraction in bovine milk and consists of two major and several minor components, and exhibits varying levels of phosphorylation (Swaisgood, 1992; Farrell Jr *et al*., 2009) and intermolecular disulfide bonding (Rasmussen *et al*., 1992, 1994). The reference protein for this family is α_{s2}-CN A-11P, a single-chain polypeptide with an internal disulfide bond with ExPASy entry name and file number CAS2_Bovin and P02663, respectively. The primary structure of α_{s2}-CN A-11P (Fig. 4.3), reported by Brignon *et al*. (1977), has been changed to Gln rather than Glu at position 87, as indicated by cDNA sequencing (Stewart *et al*., 1987) and DNA sequencing (Groenen *et al*., 1993). In addition to the aforementioned 11P variant of α_{s2}-CN A, 10P, 12P and 13P forms of this protein have also been observed (Brignon *et al*., 1976). Three centres of phosphorylation have been identified, i.e. f8–16, which contains the phosphorylated residues Ser8, Ser9, Ser10 and Ser16; f56–63, which contains the phosphorylated residues Ser56, Ser57, Ser58 and Ser61; and f126–133, which contains the phosphorylated residues Ser129 and Ser131 (De Kruif and Holt, 2003).

The primary sequence of α_{s2}-CN A-11P, as outlined in Fig. 4.3, contains two Cys residues, i.e. Cys36 and Cys40, which occur in intra- and intermolecular disulphide bonds. In α_{s2}-CN isolated from bovine milk, >85% of the protein is

1									**10**										**20**
Lys-	Asn-	Thr-	Met-	Glu-	His-	Val-	SerP-	SerP-	SerP-	Glu-	Glu-	Ser-	Ile-	Ile-	SerP-	Gln-	Glu-	Thr-	Tyr-
21									**30**										**40**
Lys-	Gln-	Glu-	Lys-	Asn-	Met-	Ala-	Ile-	Asn-	Pro-	Ser-	Lys-	Glu-	Asn-	Leu-	Cys-	Ser-	Thr-	Phe-	Cys-
41									**50**										**60**
Lys-	Glu-	Val-	Val-	Arg-	Asn-	Ala-	Asn-	Glu-	Glu-	Glu-	Tyr-	Ser-	Ile-	Gly-	SerP-	SerP-	SerP-	Glu-	Glu-
61									**70**										**80**
SerP-	Ala-	Glu-	Val-	Ala-	Thr-	Glu-	Glu-	Val-	Lys-	Ile-	Thr-	Val-	Asp-	Asp-	Lys-	His-	Tyr-	Gln-	Lys-
81									**90**										**100**
Ala-	Leu-	Asn-	Glu-	Ile-	Asn-	Gln-	Phe-	Tyr-	Gln-	Lys-	Phe-	Pro-	Gln-	Tyr-	Leu-	Gln-	Tyr-	Leu-	Tyr-
101									**110**										**120**
Gln-	Gly-	Pro-	Ile-	Val-	Leu-	Asn-	Pro-	Trp-	Asn-	Gln-	Val-	Lys-	Arg-	Asn-	Ala-	Val-	Pro-	Ile-	Thr-
121									**130**										**140**
Pro-	Thr-	Leu-	Asn-	Arg-	Glu-	Gln-	Leu-	SerP-	Thr-	SerP-	Glu-	Glu-	Asn-	Ser-	Lys-	Lys-	Thr-	Val-	Asp-
141									**150**										**160**
Met-	Glu-	Ser-	Thr-	Glu-	Val-	Phe-	Thr-	Lys-	Lys-	Thr-	Lys-	Leu-	Thr-	Glu-	Glu-	Glu-	Lys-	Asn-	Arg-
161									**170**										**180**
Leu-	Asn-	Phe-	Leu-	Lys-	Lys-	Ile-	Ser-	Gln-	Arg-	Tyr-	Gln-	Lys-	Phe-	Ala-	Leu-	Pro-	Gln-	Tyr-	Leu-
181									**190**										**200**
Lys-	Thr-	Val-	Tyr-	Gln-	His-	Gln-	Lys-	Ala-	Met-	Lys-	Pro-	Trp-	Ile-	Gln-	Pro-	Lys-	Thr-	Lys-	Val-
201									**210**										
Ile-	Pro-	Tyr-	Val-	Arg-	Tyr-	Leu													

Fig. 4.3 Amino acid sequence of α_{s2}-CN A-11P

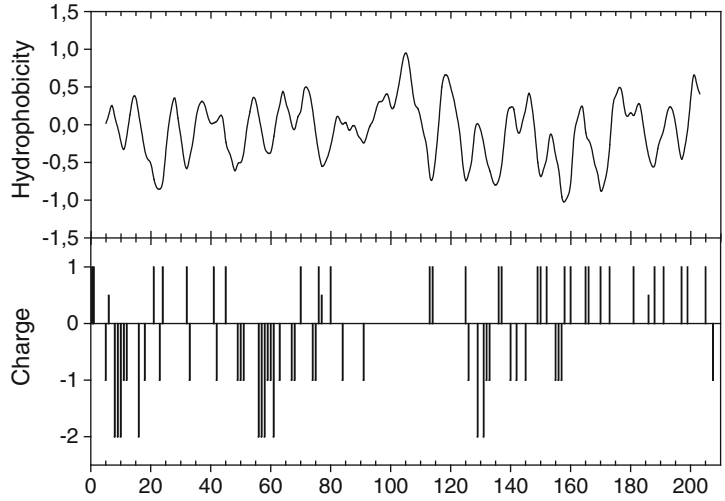

Fig. 4.4 Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of α_{s2}-CN A-11P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

in monomeric form containing the intramolecular disulphide bond, with the remaining fraction of α_{s2}-CN consisting of dimers, which can be oriented parallel or antiparallel (Rasmussen *et al.*, 1992, 1994). Brignon *et al.* (1977) pointed out that two very large segments of α_{s2}-CN, of ~80 residues, show very high sequence homology with each other and may arise from gene duplication. Sequence alignment by Farrell Jr *et al.* (2009) showed that the best homologous alignment was for residues 42–122 and 124–207. According to Farrell Jr *et al.*, (2009), the α_{s2}-CN molecule can be divided into five distinct regions. Residues 1–41 and 42–80 form typical casein phosphopeptide regions with high charge and low hydrophobicity, whereas residues 81–125 form a slightly positively charged region of high hydrophobicity and residues 126–170 form the so-called phosphopeptide analogue, with high negative charge but low phosphate content; finally, residues 171–207 have high positive charge and high hydrophobicity (Farrell Jr *et al.*, 2009). Similar trends are available from the hydrophobicity and charge distribution in Fig. 4.4.

Some properties of α_{s2}-CN are given in Table 4.4. The 207 amino acids yield a molecular mass of ~24.3 kDa, which further increases to 25.2 kDa as a result of the phosphorylation of 11 Ser residues. For the non-phosphorylated polypeptide chain of α_{s2}-CN, a pI of ~8.3 is predicted, but the aforementioned phosphorylation of 11 Ser residues decreases pI considerably to ~4.9. Because of the high level of charged residues, i.e. 33 residues able to carry a positive charge and 39 capable of carrying a negative charge, α_{s2}-CN is generally regarded as the most hydrophilic of the caseins.

4.4.2 Genetic Polymorphism of α_{s2}-Casein

The A variant of α_{s2}-CN is most frequently observed in Western breeds. The B variant was observed with low frequencies in Zebu cattle in South Africa, but a specific site of mutation for α_{s2}-CN B has not been identified to date. Variant α_{s2}-CN C was observed in yaks in the Nepalese

Table 4.4 Amino acid composition and properties of α_{s2}-CN A-11P

Amino acid	α_{s2}-CN A-11P		
Ala	8	Total residues	207
Arg	6	Positively charged residues (Lys/Arg/His)	33
Asn	14	Negatively charged residues (Glu/Asp/SerP)	39
Asp	4	Aromatic residues (Tyr/Phe/Thr)	20
Cys	2		
Gln	16	Molecular mass	
Glu	24	Based on primary structure	24,348 Da
Gly	2	Including phosphorylation	25,206 Da
His	3		
Ile	11	pI	
Leu	13	Based on primary structure	8.34
Lys	24	Including phosphorylation	4.95
Met	4		
Phe	6	Extinction coefficient at 280 nm[a]	29,005 M^{-1} cm^{-1}
Pro	10		
Ser	17	Absorbance at 1 g L^{-1} at 280 nm[a]	1.191
Thr	15		
Trp	2	Aliphatic index[a]	68.7
Tyr	12		
Val	14	Grand average of hydropathicity (GRAVY)[a]	−0.918
		$H\Phi_{ave}$ (kJ/residue)[a]	4.64

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

Table 4.5 Differences in the amino acid sequence of genetic variants of α_{s2}-casein compared to α_{s2}-CN A-11P

	Position			
Variant	33	47	51–59	130
A	*Glu*	*Ala*		*Thr*
B				
C	Gly	Thr		Ile
D			Deleted	

valley and the Republic of Mongolia (Grosclaude *et al.*, 1976, 1982). As shown in Table 4.5, the C variant differs from the A variant at positions 33, 47 and 130, where Gly, Thr and Ile replace Glu, Ala and Thr, respectively (Mahe and Grosclaude, 1982). Variant α_{s2}-CN D was observed in Vosgienne and Montbeliarde breeds (Grosclaude *et al.*, 1978) and in three Spanish breeds (Osta *et al.*, 1995). The D variant differs from α_{s2}-CN A by the deletion of nine amino acid residues from positions 51–59 (Grosclaude *et al.*, 1978), which is caused by the skipping of exon VIII, a 27-nucleotide sequence that encodes amino acid residues 51–59 (Bouniol *et al.*, 1993).

4.4.3 Secondary Structure of α_{s2}-Casein

Estimates of the secondary structure of α_{s2}-CN have been obtained using a variety of techniques and show considerable differences. Garnier *et al.* (1978) suggested 54% α-helix, 15% β-sheet, 19% turns and 13% unspecified structure, whereas Hoagland *et al.* (2001) suggested 24–32% α-helix, 27–37% β-sheet, 24–31% turns and 9–22% unspecified structure. Furthermore, Tauzin *et al.* (2003) suggested 45% α-helix, 6% β-sheet and 49% unspecified structure, whereas 15% polyproline II structure was suggested by Adzhubei and Sernberg (1993). Most recently, Farrell *et al.* (2009) suggested 46% α-helix, 9% β-sheet, 12% turns, 7% polyproline II, 19% non-continuous α-helix or β-sheet and 7% unspecified

secondary structure. Higher order structures of caseins are described in further detail in Chap. 5.

4.4.4 Association Properties of α_{s2}-Casein

Given the aforementioned amphipathic and highly charged structure of α_{s2}-CN, it is not surprising that its self-association properties strongly depend on ionic strength (Snoeren *et al.*, 1980). α_{s2}-CN associates less extensively than α_{s1}-CN, but it does exhibit consecutive self-associations, the extent of which at 20°C reaches a maximum at an ionic strength of 0.2–0.3, but decreases at higher ionic strength (Snoeren *et al.*, 1980). This perhaps unexpected decrease in association at higher ionic strengths may be due to ionic suppression of electrostatic interactions between the N-terminal and the C-terminal domains (Snoeren *et al.*, 1980). Snoeren *et al.* (1980) assumed that α_{s2}-CN particles under such conditions are spherical, which is indeed apparent from the electron micrographs reported by Thorn *et al.* (2008). However, when α_{s2}-CN is incubated at higher temperatures, e.g. 37 or 50°C, ribbon-like fibrils with a diameter of ~12 nm and length >1 μm, which occasionally form loop structures, are observed (Thorn *et al.*, 2008). The formation of such fibrillar structures is optimal at pH 6.5–6.7 and more extensive at higher temperature. The presence of α_{s1}-CN inhibits fibril formation by α_{s2}-CN, whereas the presence of β-CN has little effect on α_{s2}-CN fibril formation. Fibril formation is also reduced when the intra- and intermolecular disulphide bonds in α_{s2}-CN are disrupted by the reducing agent, dithiothreitol (Thorn *et al.*, 2008).

4.4.5 Interactions of α_{s2}-Casein with Calcium

Of the caseins, α_{s2}-casein has the highest number of phosphorylated residues and is also the most sensitive to calcium-induced precipitation. Calcium-induced precipitation of α_{s2}-CN occurs at calcium concentrations less than 2 mM (Toma and Nakai, 1973; Aoki *et al.*, 1985). As for α_{s1}-CN precipitates, calcium-induced precipitates of α_{s2}-CN are readily solubilized in 4 M urea, suggesting that no calcium-induced cross-linkage of proteins occurs and that the driving forces behind the calcium-induced interaction are driven by hydrogen bonding and hydrophobic interactions in the absence of electrostatic repulsion (Aoki *et al.*, 1985). This is further substantiated by the fact that dephosphorylation of α_{s2}-CN renders the protein insoluble at neutral pH, probably due to the low net charge on the protein at these conditions (Aoki *et al.*, 1985; Table 4.4).

4.5 β-Casein

4.5.1 Primary Structure of β-Casein

The β-CN family constitutes up to 35% of the casein of bovine milk. The reference protein for this family, β-CN A^2-5P, contains 209 residues and its ExPASy entry name and file number are CASB_Bovin and P02666, respectively. The protein was chemically sequenced by Ribadeau-Dumas *et al.* (1972), sequenced from its cDNA by Jimenez-Flores *et al.* (1987) and Stewart *et al.* (1987) and from its gene by Bonsing *et al.* (1988). The sequence for β-CN A^2-5P is shown in Fig. 4.5. This sequence was corrected from the original sequences by Yan and Wold (1984) and Carles *et al.* (1988) and differs from the original sequences at four positions: Glu for Gln at positions 117, 175 and 195 and reversal of Pro137 and Leu138. The changes at residues 117 and 175 were confirmed by both groups and by gene sequencing, whereas the reversal of residues 137 and 138 is not in agreement with cDNA-sequencing data (Jimenez-Flores et al., 1987), which is in accordance with the original data. However, the Leu-Pro substitution is a one-base change, and mutations could occur and not be observed by HPLC-mass spectroscopy (MS) of peptides or by electrophoresis of the proteins. Preference is, however, given to the two aforementioned independent protein-sequencing reports. In a similar fashion, the change at position 195 is not in agreement with the cDNA

```
 1                                  10                                  20
Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val- Pro-Gly-Glu- Ile- Val-Glu-SerP-Leu-SerP-SerP-SerP-Glu-
 21                                 30                                  40
Glu- Ser- Ile- Thr-Arg- Ile- Asn-Lys-Lys- Ile- Glu-Lys-Phe-Gln-SerP-Glu- Glu- Gln- Gln-Gln-
 41                                 50                                  60
Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys- Ile- His-Pro-Phe-Ala-Gln- Thr- Gln- Ser- Leu- Val- Tyr-
 61                                 70                                  80
Pro-Phe-Pro-Gly-Pro- Ile- Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile- Pro- Pro- Leu- Thr- Gln- Thr-
 81                                 90                                  100
Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val- Ser- Lys- Val- Lys-Glu-
101                                110                                 120
Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro- Val- Glu- Pro- Phe-Thr-
121                                130                                 140
Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-
141                                150                                 160
Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-
161                                170                                 180
Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr-
181                                190                                 200
Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-
201
Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val
```

Fig. 4.5 Amino acid sequence of β-CN A^2-5P

results, but, in this case, three other lines of evidence support the occurrence of only Glu at residue 195, i.e. the two protein-sequencing corrections noted previously, the invariance on electrophoresis of β-CN (f108–209) from the A^1, A^2 and A^3 genetic variants (Groves, 1969); and the purification from cheese of a bitter peptide *β*-CN (f193–209), the sequence of which is identical to the chemically corrected sequences (Gouldsworthy *et al.*, 1996).

Some features of β-CN A^2-5P are shown in Table 4.6, whereas the distribution of charge and hydrophobicity over the molecule is shown in Fig. 4.6. This 209 amino acid protein has a molecular mass which is increased from 23.6 kDa for the primary structure to 24.0 kDa following phosphorylation of the aforementioned five Ser residues. The pI of the non-phosphorylated amino acid is estimated at 5.1, which decreases to ~4.7 as a result of phosphorylation, which is somewhat lower than experimental values of 4.8–5.0 observed by Trieu-Cuot and Gripon (1981). Some of the unique properties of β-CN are derived from the fact that it is strongly amphipathic. The N-terminus of β-CN, residues 1–40, contains essentially all the net charge of the molecule and has a low hydrophobicity and contains only two Pro residues. This section also contains the five phosphorylated Ser residues, i.e. Ser_{15}, Ser_{17}, Ser_{18}, Ser_{19} and Ser_{35}, of which the first four form a centre of phosphorylation (De Kruif and Holt, 2003). The middle section of β-CN, i.e. residues 41–135, contains little charge and moderate hydrophobicity, whereas the C-terminal, section 136–209, contains many of the apolar residues and is characterized by little charge and high hydrophobicity.

4.5.2 Genetic Polymorphism of β-Casein

In addition to the aforementioned A^2 variant of β-CN, a number of other genetic variants have been observed. The amino acid substitutions giving rise to all variants of β-CN are given in Table 4.7. In addition, Chung *et al.* (1995) identified variant A^4 in native Korean cattle using electrophoresis only; its substitutions compared to the A^2 reference protein are thus far unknown. The A^1 variant of β-CN differs from the A^2 variant only by the substitution at position 67 of His for Pro (Bonsing *et al.*, 1988), whereas the A^3 variant contains Gln instead of His at position 106 (Ribadeau-Dumas *et al.*, 1970). In addition, β-CN B contains the aforementioned mutation for

Table 4.6 Amino acid composition and properties of β-CN A^2-5p

Amino acid	β-CN A^2-5P		
Ala	5	Total residues	209
Arg	4	Positively charged residues (Lys/Arg/His)	20
Asn	5	Negatively charged residues (Glu/Asp/SerP)	28
Asp	4	Aromatic residues (Tyr/Phe/Thr)	14
Cys	0		
Gln	20	Molecular mass	
Glu	19	Based on primary sequence	23,583 Da
Gly	5	Including phosphorylation	23,973 Da
His	5		
Ile	10	pI	
Leu	22	Based on primary sequence	5.13
Lys	11	Including phosphorylation	4.65
Met	6		
Phe	9	Extinction coefficient at 280 nm[a]	11,460 M^{-1} cm^{-1}
Pro	35		
Ser	16	Absorbance at 1 g L^{-1} at 280 nm[a]	0.486
Thr	9		
Trp	1	Aliphatic index[a]	88.5
Tyr	4		
Val	19	Grand average of hydropathicity (GRAVY)[a]	−0.355
		$H\Phi_{ave}$ (kJ/residue)[a]	5.58

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

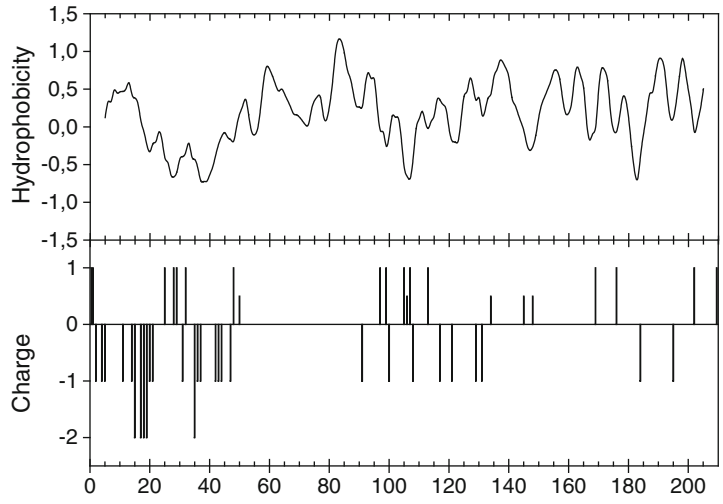

Fig. 4.6 Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of β-CN A^2-5P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

Table 4.7 Differences in the amino acid sequence of genetic variants of β-casein compared to β-CN A^2-5P

	Position													
Variant	18	25	35	36	37	67	72	88	93	106	122	137/138	152	?
A^1						His								
A^2	*SerP*	*Arg*	*SerP*	*Glu*	*Glu*	*Pro*	*Gln*	*Leu*	*Met*	*His*	*Ser*	*Leu/Pro*	*Pro*	*Gln*
A^3										Gln				
B						His					Arg			
C			Ser		Lys	His								
D	Lys													
E				Lys										
F						His							Leu	
G						His						Leu		
H^1		Cys						Ile						
H^2							Glu		Leu					Glu
I									Leu					

the A^1 variant, as well as Arg for Ser at position 122 (Grosclaude *et al.*, 1974a). Likewise, β-CN C is also a variant of β-CN A^1, which is not phosphorylated at Ser_{35} and contains Lys instead of Glu at position 37. β-CN D differs from β-CN A^2 only at position 18, whereas it contains Lys instead of a phosphorylated Ser residue, whereas β-CN E contains Lys instead of Glu at position 36 (Grosclaude *et al.*, 1974b). Visser *et al.* (1995) identified β-CN F, which contains the A^1 substitution in addition to Leu for Pro at residue 152. Dong and Ng-Kwai-Hang (1998) identified β-CN G-5P, which is similar to β-CN A^1 and F but contains a Leu in place of Pro at either position 137 or 138, depending on the sequence assigned, as the Pro-Leu reversal, as outlined above, is controversial. Han *et al.* (2000) showed that β-CN H^1 represents two substitutions relative to the corrected reference β-CN A^2, i.e. Arg to Cys at position 25 and Leu to Ile at position 88. A genetic variant, discovered by Senocq *et al.* (2002), was termed β-CN H^2, which differs from the A^2 variant at two known positions, i.e. Leu instead of Met at position 93 and Glu instead of Gln at position 72; in addition, a substitution of Gln to Glu occurs somewhere between residues 114 and 169 but was not located (Senocq *et al.*, 2002). Finally, the I variant of β-CN was described by Jann *et al.* (2002) and contains only the Leu for Met substitution of the H^2 variant at position 93.

4.5.3 Secondary Structure of β-Casein

Originally, β-CN was predicted to have little or no secondary structure and, with the exception of 10% α-helix, was predicted to occur as a random coil (Herskovits, 1966; Noelken and Reibstein, 1968), which was further supported by the results of Caessens *et al.* (1999). The presence of α-helix structure in β-CN was further shown by Creamer *et al.* (1981), Graham *et al.* (1984), Farrell Jr *et al.* (2001) and Qi *et al.* (2004, 2005), with values ranging from 7 to 25%. However, 15–33% β-sheet structure was also reported to be present in β-CN, as well as 20–30% turns (Creamer *et al.*, 1981; Graham *et al.*, 1984; Farrell Jr *et al.*, 2001; Qi *et al.*, 2004, 2005). Using optical rotary dispersion analysis, Garnier (1966) suggests that polyproline II could be an important feature in β-casein structure. Subsequent studies have indeed confirmed the presence of 20–25% polyproline II structure in β-CN (Farrell Jr *et al.*, 2001; Syme *et al.*, 2002; Qi *et al.*, 2004). Higher order structures of caseins are dealt with in detail in Chap. 5.

4.5.4 Association Properties of β-Casein

The presence of distinct polar and hydrophobic domains in β-CN clearly manifests itself in the extremely temperature-dependent self-association behaviour of β-CN. At 0–4°C, primarily monomers of β-CN are observed (Payens and Van Markwijk, 1963), but even under these conditions, polymeric structure is not entirely absent (Farrell Jr *et al*., 2001). The hydrodynamic behaviour of β-CN under these conditions approaches that of a random coil, with the Stokes radius of 3.7 nm, determined by gel chromatography (Schmidt and Payens, 1972), agreeing well with values obtained by sedimentation and viscosity, and is also consistent with the 4–5-nm size of spherical particles observed by electron microscopy (Andrews *et al*., 1979). Small angle X-ray scattering indicates a radius of gyration of 4.6 nm (Schmidt and Payens, 1972, Andrews *et al*., 1979).

As the temperature is increased above 4–5°C, β-CN undergoes a highly cooperative, reversible, rapidly equilibrating discrete self-association, yielding large polymers with a narrow size distribution (Payens and Van Markwijk, 1963; Payens and Heremans, 1969; Payens *et al*., 1969; Schmidt and Payens, 1972; Niki *et al*., 1977; Andrews *et al*., 1979; Arima *et al*., 1979; Buchheim and Schmidt, 1979; Evans and Phillips, 1979; Takase *et al*., 1980; Schmidt, 1982; Thurn *et al*., 1987; Kajiwara *et al*., 1988; Leclerc and Calmettes, 1997a, b, 1998; Farrell Jr *et al*., 2001; De Kruif and Grinberg, 2002; O'Connell *et al*., 2003; Qi *et al*., 2004, 2005; Gagnard *et al*., 2007). The properties for this monomer–polymer equilibrium can be treated using a shell model for the polymer micelle with a continuous distribution of intermediates between the monomer and largest polymer micelle (Tai and Kegeles, 1984; De Kuif and Grinberg, 2002; O'Connell *et al*., 2003; Mikheeva *et al*., 2003). There appears to be a critical concentration above which micelles are formed, ranging from less than 0.5 mg/mL to about 2 mg/mL (Schmidt and Payens, 1972; Niki *et al*., 1977; Evans *et al*., 1979), which depends on the temperature, ionic strength and pH. The size of the polymer micelle has been characterized by the number of monomers in the polymer, estimates of which have been shown to vary from 15 to 60 (Schmidt and Payens, 1972; Buchheim and Schmidt, 1979; Takase *et al*., 1980; Thurn *et al*., 1987; Kajiwara *et al*., 1988; Farrell Jr *et al*., 2001); the radius of gyration, with varying estimates of 7.3–13.5 nm (Andrews *et al*., 1979; Thurn *et al*., 1987; Kajiwara *et al*., 1988); the Stokes radius of ~15 nm (Niki *et al*., 1977; Thurn *et al*., 1987) and the radius observed by electron microscopy of 8–17 nm (Arima *et al*., 1979; Buchheim and Schmidt, 1979). Increasing ionic strength shifts the equilibrium towards the polymer micelle but affects the number of monomers in the micelle only slightly (Schmidt and Payens, 1972; Takase *et al*., 1980), whereas increasing the temperature shifts the equilibrium position and increases the number of monomers in the micelle (Takase *et al*., 1980). In the theoretical ratio, radius of gyration/Stokes radius is 0.775 for a hard sphere (Thurn *et al*., 1987; Kajiwara *et al*., 1988), while that observed for the β-CN polymer micelle is less than 0.6, suggesting the immobilization of water in a soft outer layer surrounding a more dense core (Kajiwara *et al*., 1988).

Removal of the C-terminal three hydrophobic residues, Ile-Ile-Val, greatly reduces the association (Thompson *et al*., 1967; Evans and Phillips, 1979), as does removal of the C-terminal 17 amino acids (Qi *et al*., 2005). Removal of these 17 amino acids (Qi *et al*., 2005) or the 20 C-terminal amino acids (Berry and Creamer, 1975) renders β-CN virtually incapable of binding the hydrophobic surface probe ANS. The importance of hydrophobic interactions in the micellization of β-CN is further exemplified by the enhanced micellization when H_2O is replaced by D_2O (Evans and Phillips, 1979) or when ethanol is added (Mikheeva *et al*., 2003) and by the reduced micellization of β-CN in the presence of urea (Mikheeva *et al*., 2003). The importance of charges on the N-terminus on the micellization of β-CN is strongly impaired by the absence of post-translational phosphorylation but this loss of micellization is partially restored by duplication of the 6 N-terminal amino acids of β-CN in expression (Gagnard *et al*., 2007).

4.5.5 Interactions of β-Casein with Calcium and Other Cations

Compared to α_{s1}-CN and α_{s2}-CN, β-CN is less sensitive to calcium-induced precipitation. At 37°C, β-CN precipitates in the range of 8–15 mM Ca^{2+} at 37°C (Schmidt, 1969; Parker and Dalgleish, 1981; Farrell Jr *et al*., 1988). However, at 1°C, β-CN remains in solution at concentrations up to 400 mM $CaCl_2$ (Farrell Jr *et al*., 1988). Under physiological conditions, β-CN is capable of binding approximately seven calcium ions per molecule (Parker and Dalgleish, 1981; Baumy and Brule, 1988). Binding of calcium by β-CN increases with increasing temperature, whereas an increase in ionic strength reduces the binding of calcium by β-CN (Parker and Dalgleish, 1981; Baumy and Brule, 1988). In addition, the binding of calcium by β-CN decreases with decreasing pH (Baumy and Brule, 1988). The binding of other di- and trivalent cations has also been studied; binding of magnesium, zinc and manganese shows comparable dependence on pH and ionic strength to the binding of calcium, whereas the binding of iron and copper by β-CN is virtually independent of pH and ionic strength (Baumy and Brule, 1988). The amount of calcium required to induce precipitation of β-CN decreases strongly with increasing temperature, whereas decreases in the amount of calcium bound by β-CN at the point of precipitation are also observed (Parker and Dalgleish, 1981). Both dephosphorylation and glycation of β-casein have been shown to improve the stability of β-casein to calcium-induced precipitation (Darewicz *et al*., 1999).

4.6 κ-Casein

4.6.1 Primary Structure of κ-Casein

Within the caseins, κ-CN displays some rather unique features. It is the smallest of the caseins, is has a low level of phosphorylation, has a low sensitivity to calcium and is the only one of the caseins to occur in glycosylated form. The primary sequence of the 169 amino acid κ-CN A 1P, which is the parent protein of the κ-CN family and has the ExPASy entry name CASK_Bovin and file accession number P02668, is shown in Fig. 4.7. Like for the other caseins, variable degrees of phosphorylation have also been found for κ-CN. The monophosphorylated form of κ-CN appears to be phosphorylated exclusively at Ser_{149}, whereas the diphosphorylated form of κ-CN is phosphorylated at Ser_{149} and Ser_{121} (Mercier, 1981; Minkiewicz *et al*., 1996; Talbo *et al*., 2001; Holland *et al*., 2006). For the triphosphorylated form of κ-CN,

1									**10**										**20**
Gln-	Glu-	Gln-	Asn-	Gln-	Glu-	Gln-	Pro-	Ile-	Arg-	Cys-	Glu-	Lys-	Asp-	Glu-	Arg-	Phe-	Phe-	Ser-	Asp-
21									**30**										**40**
Lys-	Ile-	Ala-	Lys-	Tyr-	Ile-	Pro-	Ile-	Gln-	Tyr-	Val-	Leu-	Ser-	Arg-	Tyr-	Pro-	Ser-	Tyr-	Gly-	Leu-
41									**50**										**60**
Asn-	Tyr-	Tyr-	Gln-	Gln-	Lys-	Pro-	Val-	Ala-	Leu	Ile-	Asn-	Asn-	Gln-	Phe-	Leu-	Pro-	Tyr-	Pro-	Tyr-
61									**70**										**80**
Tyr-	Ala-	Lys-	Pro-	Ala-	Ala-	Val-	Arg-	Ser-	Pro	Ala-	Gln-	Ile-	Leu-	Gln-	Trp-	Gln-	Val-	Leu-	Ser-
81									**90**										**100**
Asn-	Thr-	Val-	Pro-	Ala-	Lys-	Ser-	Cys-	Gln-	Ala	Gln-	Pro-	Thr-	Thr-	Met-	Ala-	Arg-	His-	Pro-	His-
101									**110**										**120**
Pro-	His-	Leu-	Ser-	Phe-	Met-	Ala-	Ile-	Pro-	Pro	Lys-	Lys-	Asn-	Gln-	Asp-	Lys-	Thr-	Glu-	Ile-	Pro-
121									**130**										**140**
Thr-	Ile-	Asn-	Thr-	Ile-	Ala-	Ser-	Gly-	Glu-	Pro	Thr-	Ser-	Thr-	Pro-	Thr-	Thr-	Glu-	Ala-	Val-	Glu-
141									**150**										**160**
Ser-	Thr-	Val-	Ala-	Thr-	Leu-	Glu-	Asp-	SerP-	Pro	Glu-	Val-	Ile-	Glu-	Ser-	Pro-	Pro-	Glu-	Ile-	Asn-
161																			
Thr-	Val-	Gln-	Val-	Thr-	Ser-	Thr-	Ala-	Val											

Fig. 4.7 Primary amino acid sequence of κ-CN A-1P

Table 4.8 Amino acid composition and properties of κ-CN A-1P

Amino acid	κ-CN A-1P		
Ala	14	Total residues	169
Arg	5	Positively charged residues (Lys/Arg/His)	17
Asn	8	Negatively charged residues (Glu/Asp/SerP)	28
Asp	4	Aromatic residues (Tyr/Phe/Thr)	14
Cys	2		
Gln	15	Molecular mass	
Glu	12	Based in primary sequence	18,974 Da
Gly	2	Including phosphorylation	19,052 Da
His	3		
Ile	12	pI	
Leu	8	Based on primary sequence	5.93
Lys	9	Including phosphorylation	5.60
Met	2		
Phe	4	Extinction coefficient at 280 nm[a]	19035 M^{-1} cm^{-1}
Pro	20		
Ser	13	Absorbance at 1 g L^{-1} at 280 nm[a]	1.003
Thr	15		
Trp	1	Aliphatic index[a]	73.3
Tyr	9		
Val	11	Grand average of hydropathicity[a]	–0.557
		$H\Phi_{ave}$ (kJ/residue)[a]	5.12

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

Holland *et al*. (2006) recently reported that the additional amino acid residue to be phosphorylated is not a Ser residue, but Thr145.

Some features of κ-CN A-1P are shown in Table 4.8, whereas the distribution of hydrophobicity and charge over the protein chain are shown in Fig. 4.8. Based on the amino acid sequence, it can be deduced that of the 169 amino acids, 17 can be positively charged, whereas 28 can be negatively charged and there are a further 14 aromatic residues. Both hydrophobicity and charge are distributed unevenly throughout the protein (Fig. 4.8). Negative charges are found only in the N-terminal fragment 1–20 and the C-terminal fragment 115–169; the intermittent fragment 21–114 is devoid of negatively charged residues. Additional negative charges arising from phosphorylation are also in the C-terminal segment 115–169, as would be negative charges arising from glycosylation, which, as discussed later, can occur on six Thr residues in this segment. Positive charges can be found in the N-terminal segment 1–116, but not in the C-terminal segment 117–169. Hydrophobicity distributions highlight, as for charges, an uneven distribution of hydrophobicity throughout κ-CN. Segment 1–20 shows predominantly hydrophilic behaviour, whereas segment 21–110 contains some strongly hydrophobic patches, which is in agreement with the absence of negatively charged and a low number of positively charged residues in this segment. Segment 110–120 is strongly hydrophilic, whereas the remainder, i.e. segment 121–169 shows some hydrophilic and hydrophobic areas. It should be noted that post-translational phosphorylation and glycosylation occurring in this part of the protein will reduce hydrophobicity considerably.

Not taking into account post-translational modification, the molecular mass of κ-CN A was reported as 19.0 kDa. Increases in mass arise from post-translational phosphorylation and glycosylation. Based on the amino acid sequence, a pI for κ-CN A of ~5.9 can be expected. However,

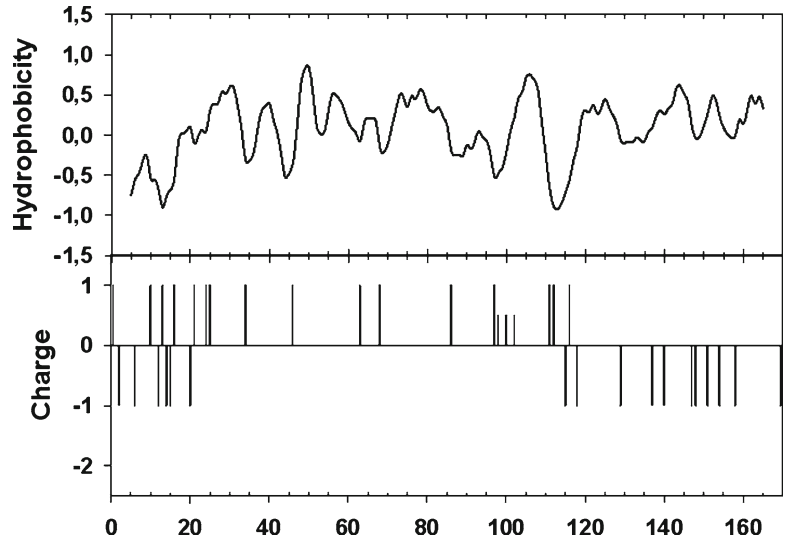

Fig. 4.8 Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of κ-CN A-1P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

experimental observations have shown considerably lower values for the isoelectric point of κ-CN, as low as pH 3.5 (Holland *et al*., 2006), which is due to increased negative charges on the protein arising from post-translational phosphorylation and glycosylation. For the non-glycosylated monophosphorylated variants of κ-CN A and B, pI values of 5.56 and 5.81 were found by two-dimensional electrophoresis, with consistent reductions in pI apparent with increasing degree of phosphorylation and glycosylation (Holland *et al*., 2004).

4.6.2 Genetic Variation of κ-Casein

κ-CN A predominates in Western breeds, with the exception of Jerseys (Thompson and Farrell Jr, 1974; Bech and Kristiansen, 1990; Ng-Kwai-Hang and Grosclaude, 2003). In addition, a number of other variants of κ-CN have also been identified (Table 4.9). The major other variant of κ-CN is κ-CN B, which differs from κ-CN A by substitution at position 136 of Ile for Thr and at position 148 of Ala for Asp (Mercier *et al*., 1973). The C variant of κ-CN differs from κ-CN A by substitution of His for Arg at position 97 (Miranda *et al*., 1993). The E variant of κ-CN arises from a substitution at position 155, i.e. Gly for Ser (Miranda *et al*., 1993). κ-CN F^1 was discovered in both Zebu and Black and White hybrid cattle and contains Val instead of Asp at position 148 (Sulimova *et al*., 1992). κ-CN F^2 was reported to be a variant of κ-CN B, containing His instead of Arg at position ten (Prinzenberg *et al*., 1996). Erhardt *et al*. (1996) reported the occurrence of κ-CN G^1 in alpine breeds, which, in addition to the substitutions occurring for κ-CN B, also contains Cys instead of Arg at position 97. κ-CN G^2 was shown to occur in the milk of *Bos grunniens* and was shown to contain Ala instead of Asp at position 148. In Pinzgauer cattle, Prinzenberg *et al*. (1999) identified κ-CN H, which differed from κ-CN A by an Ile for Thr substitution at position 135. In another study, Prinzenberg *et al*. (1999) described κ-CN I,

Table 4.9 Differences in the amino acid sequence of genetic variants of κ-casein compared to κ-CN A-1P

	Position						
Variant	10	97	104	135	136	148	155
A	*Arg*	*Arg*	*Ser*	*Thr*	*Thr*	*Asp*	*Ser*
B					Ile	Ala	
C		His					
E							Gly
F^1						Val	
F^2	His				Ile	Ala	
G^1		Cys			Ile	Ala	
G^2						Ala	
H					Ile		
I			Ala				
J					Ile	Ala	Arg

which differs from κ-CN A by Ala for Ser substitution at position 104. Finally, Mahe *et al*. (1999) described the occurrence of κ-CN J, which seems to have arisen from an Arg for Ser mutation at position 155 in *Bos taurus* cattle on the Ivory Coast. As outlined previously, however, κ-CN A and B predominate strongly in Western breeds of cattle.

From a technological perspective, the Phe105-Met106 bond in κ-CN is extremely important, as it is the hydrolysis of this bond by chymosin, or proteinases with comparable specificity, that initiates the gelation of milk, which will ultimately be processed into a cheese curd and a ripened or unripened cheese. The N-terminal segment 1–105 arising from the chymosin-induced hydrolysis of κ-CN is called *para*-κ-CN, whereas the C-terminal fragment 106–169 is called the caseinomacropeptide (CMP); when CMP is glycosylated, it is often referred to as glycomacropeptide (GMP). From Table 4.9, it is apparent that this sequence is conserved in all genetic variants of κ-CN. However, for κ-CN I, the adjoining Ser104 residue is replaced by the considerably more hydrophobic Ala residue. It is also worthwhile noticing that, as outlined further in later stages, all post-translational modifications of κ-CN occur in the CMP segment of the molecule.

4.6.3 Glycosylation of κ-Casein

Of the caseins, κ-CN is the only one for which post-translational glycosylation has been shown to occur. Vreeman *et al*. (1986) observed that ~40% of κ-CN is non-glycosylated, whereas the remainder can contain up to six glycans. Glycosylation sites in κ-CN were found to be the Thr residues at positions 121, 131, 133, 142, 145 and 165 (Pisano *et al*., 1994; Molle and Leonil, 1995; Minkiewicz *et al*., 1996). Holland *et al*. (2004, 2005, 2006) showed that the different glycoforms of κ-CN can be separated readily by 2D electrophoresis on the basis of isoelectric point and molecular mass, yielding up to 16 different spots for κ-CN with isoelectric points down to ~3.5. Such separations have laid the basis for the recent elucidation of the glycosylation pattern of κ-CN. Using tandem MS sequencing of chemically tagged peptides, it was observed that the mono-glycoform of κ-CN was glycosylated exclusively at Thr131, the di-glycoform exclusively at Thr131 and Thr142 and the tri-glycoform at Thr131, Thr133 and Thr142 (Holland *et al*., 2005). The tetra-glycoform of κ-CN B was shown to be glycosylated at Thr145, in addition to the three already-mentioned glycosylation sites, Thr131, Thr133 and Thr142 (Holland *et al*., 2006). The remaining two glycosylation sites of κ-CN were not confirmed by Holland *et al*. (2006) but are most likely, as proposed by Pisano *et al*. (1994) and Minkewicz *et al*. (1996), to be Thr121 and Thr165. In general, κ-CN B appears to be more heavily glycosylated than κ-CN A, also displaying a more complex and variable glycosylation pattern (Coolbear *et al*., 1996).

A variety of glycans have been shown to be attached to κ-CN, all of which have been shown to be attached to Thr residues. These glycans consist of galactose (Gal), *N*-acetylglucosamine (GalNAc) and *N*-acetyl neuraminic acid (NANA). The monosaccharide GalNac, the disaccharide Galβ(1–3)GalNac, the trisaccharides NANAcα(2–3)Galβ(1–3)GalNAc and Galβ(1–3)

[NANAcα(2–6)]GalNac and the tetrasaccharide NANAcα(2–3)Gal1β(1–3)[NANAcα(2–6)] GalNac have been identified attached to κ-CN. Saito and Itoh (1992) estimated the presence of 56.0% tetrasaccharide, 18.5% branched trisaccharide, 18.4% linear trisaccharide, 6.3% disaccharide and 0.8% monosaccharide.

4.6.4 Disulphide-Bonding Patterns of κ-Casein

The presence of the two Cys residues in κ-CN, i.e. Cys11 and Cys88, creates a complex disulphide-bonding pattern between κ-CN molecules in bovine milk. Swaisgood *et al*. (1964) showed that κ-CN obtained without reduction was apparently randomly cross-linked by intermolecular disulphide bonds, to give oligomers, with the smallest detectable oligomer having a mass of ~60 kDa, corresponding to a trimer. The existence of disulphide-cross-linked oligomers has since been substantiated (Talbot and Waugh, 1970; Farrell Jr *et al*., 1988; Groves *et al*., 1992), with the further suggestion that, during biosynthesis, reduced monomers first interact with the calcium-sensitive caseins to form micelles, followed by random cross-linking by oxidation (Pepper and Farrell Jr, 1982). In κ-CN isolated from bovine milk, only ~10% of total κ-CN appears to be in the monomeric form (Farrell Jr *et al*., 1996).

Both disulphide-cross-linked oligomers and reduced κ-CN are capable of forming polymer micelles and stabilizing calcium-sensitive caseins (Talbot and Waugh, 1970; Vreeman, 1979). In the monomeric form of κ-CN, Cys11 and Cys88 form an intramolecular disulphide bond. However, κ-CN complexes arising to octamers and larger have also been found in bovine milk. These complexes contain an apparently random distribution of disulphides, i.e. Cys11 to Cys88, Cys11 to Cys11, Cys88 to Cys11 and Cys88 to Cys88. Whether these patterns remain after isolation of the κ-CN from milk is strongly dependent on the physicochemical conditions of isolation. Particularly the presence of reducing agents such as β-mercaptoethanol and dithiothreitol will significantly impact the oligomeric distributions of κ-CN. As outlined in Table 4.9, κ-CN G^1 even contains a third Cys residue, i.e. Cys97. The impact hereof on the disulphide-bonding pattern has, however, not been studied to date.

4.6.5 Secondary Structure of κ-Casein

The secondary structure of κ-CN has been studied using a number of methods. NMR studies by Rollema *et al*. (1988) suggest a high degree of flexibility, particularly in the macropeptide part of κ-CN. Some structure, however, has been detected for κ-CN using spectroscopic methods such as FTIR and CD. Estimates suggest that κ-CN may contain 10–20% α-helix, 20–30% β-structure and 15–25% turns (Byler and Susi, 1986; Griffin *et al*., 1986; Ono *et al*., 1987; Kumosinski *et al*., 1991, 1993; Sawyer and Holt, 1993; Farrell Jr *et al*., 1996, 2003). The degree of estimated α-helical structure in κ-CN increases with increasing temperature (10–70°C), while the proportion of β-structure and turns decreases with temperature (Farrell *et al*., 2003). In addition, analysis in the presence of alcohols also results in a higher degree of α-helix in κ-CN. Several structural motifs have also been suggested, including possible antiparallel and parallel β-sheets or βαβ structure in the hydrophobic domain (Raap *et al*., 1983) and a β-turn-β-strand-β-turn motif centred on the chymosin-sensitive Phe_{105}-Met_{106} region (Creamer *et al*., 1998). The latter motif appears to be conserved in κ-CN from various species, as would be expected for specific sensitivity to aspartyl proteinases (Holt and Sawyer, 1988). Using a Raman optical activity study, Syme *et al*. (2002) identified the presence of polyproline II helical confirmation in κ-CN. Some of the predicted structure occurs in the polar macropeptide domain but the stability of ordered structure in a region of such high net charge and apparent hydration would seem questionable and contradicts the great deals of flexibility; this part of the molecule was found to exhibit in the NMR studies by Rollema *et al*. (1988).

4.6.6 Association Behavior of κ-Casein

When isolated from milk, κ-CN occurs in the form of multimeric complexes. Analysis by analytical ultracentrifugation suggests that the weight average molecular weight of these complexes is ~1,180 kDa at 25°C and ~1,550 kDa at 37°C (Groves *et al*., 1998). Electron microscopy studies have shown a radius of 5.0–7.5 nm, 9–10 nm (Parry and Carroll, 1969) or 8.9 nm (Farrell Jr *et al*., 1996). Similar values have been observed by gel permeation chromatography (9.4 nm; Pepper and Farrell Jr, 1982), dynamic light scattering (9.6 nm; Farrell Jr *et al*., 1996) and small angle neutron scattering (SANS), for which values for values of a radius of 7.4 nm (Thurn *et al*., 1987) and 8 nm (De Kruif *et al*., 2002) have been reported. Micelle size, structure and interaction radius were found to be independent of protein concentration (De Kruif *et al*., 2002). Both calcium and iron have been found to be present in isolated κ-CN, and their chelation by EDTA has been reported to result in disruption of the κ-CN particle, with subsequent aggregation into particles with a considerably broader size distribution (Farrell Jr *et al*., 1996).

Reduction of the disulphide bridges in aforementioned κ-CN particles leads to amphipathic monomers which can, like β-CN, associate into micellar structures; unlike the micellization of β-CN, micellization of reduced κ-CN shows no strong temperature dependence (Swaisgood *et al*., 1964; Vreeman *et al*., 1981). This suggests that micellization of reduced κ-CN is less dominated by hydrophobic interactions than micellization of β-CN. For the monomer–polymer micelle equilibrium of reduced κ-CN, the critical micelle concentration varies from 0.53 at an ionic strength of 0.1–0.24 mg/mL at an ionic strength of 1.0 (Vreeman, 1979; Vreeman *et al*., 1977, 1981). The degree of polymerization has been estimated at ~30 κ-CN molecules per micelle, yielding a molecular mass of ~570–600 kDa (Vreeman, 1979; Vreeman *et al*., 1981, 1986) and an estimated diameter of 23 nm (Vreeman *et al*., 1981). Such results are in agreement with values derived from SANS measurements on reduced κ-CN micelles; such measurements led De Kruif and May (1991) to conclude that reduced κ-CN micelles are spherical and consist of a dense core of ~6–7 nm, surrounded by a more open outer layer, protruding up to 14.7 nm from the centre of the core. The interactions between micelles of reduced κ-CN can be described as that of the so-called hard spheres (De Kruif and May, 1991).

When reduced and carboxymethylated κ-CN was incubated at 37°C, it was observed in addition to spherical particles, there was also a high proportion of fibrillar structures present (Farrell Jr *et al*., 2003). The formation of such fibrillar structures, with a diameter of 10–12 nm and lengths up to 600 nm, was subsequently shown to occur for native, reduced and carboxymethylated κ-CN (Thorn *et al*., 2005; Ecroyd *et al*., 2008, 2010; Leonil *et al*., 2008). When native κ-CN is used, it is the dissociated form that is involved in fibril formation (Ecroyd *et al*., 2010). Fibril formation, which has been shown to result in an increased proportion of β-sheet structure (Ecroyd *et al*., 2008; Leonil *et al*., 2008), is more extensive at higher temperature (Thorn *et al*., 2005) and is more extensive for non-glycosylated κ-CN than for its glycosylated counterpart (Leonil *et al*., 2008). The presence of α_s-CNs or β-CN inhibits fibril formation (Thorn *et al*., 2005; Leonil *et al*., 2008), whereas BSA does not inhibit fibril formation (Thorn *et al*., 2005). Segment Tyr25-Lys86 of κ-CN appears to be incorporated into the protease-resistant core of the fibrils (Ecroyd *et al*., 2008) whereas fragment 106–169, i.e., the macropeptide, in either glycosylated or non-glycosylated form, does not form fibrils under comparable circumstances (Leonil *et al*., 2008).

4.6.7 Interactions of κ-Casein with Calcium

Compared to the other caseins, interactions of calcium with κ-CN have studied far less. This is probably due to the fact that κ-CN is, unlike α_{s1}-CN, α_{s2}-CN and β-CN, the so-called calcium insensitive, i.e., it is not precipitated in the pres-

ence of excess calcium. Ono *et al*. (1980), studying the binding of calcium to κ-CN, observed that binding of calcium to phosphorylated Ser residues reached a plateau at 1 mM $CaCl_2$, whereas binding of calcium by carboxyl groups increased linearly up to 3 mM $CaCl_2$ and more slowly at higher concentrations. Spectra obtained from circular dichroism and UV analysis indicate that the binding of calcium to κ-CN does not induce changes in the secondary structure of the protein (Ono *et al*., 1980). Given the aforementioned potential role of calcium in stabilizing κ-CN particles isolated from milk (Farrell *et al*., 1996), further study on the interactions of calcium with κ-casein appears warranted.

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Higher Order Structures of the Caseins: A Paradox?

5

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5.1 Introduction and Historic Views of Casein Structure

One of the fundamental theorems of modern protein chemistry, the Anfinsen hypothesis, is that the vast majority of protein architecture in biological systems arises from the primary sequences of the proteins (Anfinsen, 1973). While the most recent discoveries of the action of chaperonins have indicated a role for these proteins in the kinetics of protein folding, the fundamental theorem still applies. A major corollary to this theorem also appears to be time tested: biological function arises from protein or nucleic acid structure.

From all of the concepts regarding casein structure-function which have been set forth over the years, two fundamental functions of casein can be envisioned:

1. The effective transport of calcium
2. The self-associations which lead to the colloidal state (Farrell *et al.*, 2002a, 2006a)

This review will concentrate on the latter function. Although casein has been studied for many years, the molecular structural basis for its function in self-association reactions in milk has been elusive. Historically, optical rotatory dispersion data from our laboratory demonstrated a lack of α-helix in the caseins, and since that was all that could be measured at the time, caseins were considered to be the model for random-coil proteins (Farrell, 1988). However, as sequences became available and circular dichroism (CD) was employed as a tool for protein analysis (Creamer *et al.*, 1981), the possibility of periodic structure was considered. Swaisgood (1982) was perhaps the first to suggest that the caseins were neither globular nor random-coil proteins and that they could be composed of rather distinct functional domains. The next important step on the road to the understanding of casein structure may be the concept of Holt and Sawyer (1993) who suggested that caseins are rheomorphic in nature. In this instance, the "formed under flow" hypothesis suggests that casein structure is not fixed at all in the absence of calcium. In its extreme, this hypothesis may be considered as the "spaghetti plate" hypothesis, in that no regular structures occur until aggregates are formed in response to calcium-phosphate binding. Supporting this hypothesis was the observation of Paulsson and Dejmek (1990) that pure caseins, when studied by differential scanning calorimetry (DSC), showed flat endotherms on heating. The cooperative unfolding of native globular proteins always yields a rather characteristic Gaussian pattern when studied by this methodology. However, Paulsson and Dejmek (1990) suggested an alternative view, i.e., caseins exhibit no peak because they contain heat-stable structures.

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The concept that caseins have little or no fixed structure seemed quite appealing, but data which we had collected in collaboration with the late Heino Susi appeared to be at odds with this concept (Byler *et al.*, 1988). We had applied Susi's Raman methodologies, which he had developed for globular proteins, to purified caseins in the absence of calcium. Inspection of the data revealed that all of the caseins had intricate amide I profiles, similar to those obtained for globular proteins, with a moderate distribution of various periodic secondary structures; FTIR analyses of the caseins are in agreement with these data. The patterns of all purified caseins and their mixtures showed bands characteristic of reasonable amounts of β-turns and β-sheet and a modest amount of α-helix. It has, therefore, been difficult to reconcile the concept of fixed structure giving rise to function with either the random-coil or the rheomorphic hypotheses.

Starting about 1990, we began to conduct a series of three-dimensional (3D) molecular modeling experiments on caseins. In these studies, we attempted to derive structures for caseins from the basic Anfinsen hypothesis. Because an infinite number of potential structures are available in conformational space to proteins the size of the caseins (≈200 residues), we attempted to arrive at working models by constraining the computer experiments to predicted secondary structures derived from primary sequence data (Garnier *et al.*, 1978). We further constrained the global structure by requiring that it conform to Raman and FTIR limits. Thus, for example, the limit for α-helix was no more than 10% and the limit on extended structure 30%. Finally, the number of turns was increased from algorithm predictions to correlate the spectroscopic data with the relatively high abundance of proline in the caseins. This was done because proline, while a structure-breaking residue for helix and sheet, can be instrumental in the formation of turns in peptides and proteins (Ananthanarayanan *et al.*, 1984; Cohen *et al.*, 1986). Using these principles in conjunction with force field calculations, we arrived at refined energy-minimized working models for κ-, β-, and α_{S1}-caseins which are shown in Fig. 5.1 (Kumosinski *et al.*, 1993a, b, 1994a). At a later time, it was discovered that α_{S2}-casein was structurally similar to the chloride channel (CLIC) proteins which have known crystal structures (Farrell *et al.*, 2009). This discovery allowed us to produce a 3D structure for α_{S2}-casein by homologous modeling techniques (Farrell *et al.*, 2009), and it is also shown in Fig. 5.1.

5.2 New Views of Protein Structure

Historically, proteins were thought to fold or unfold in a concerted fashion as shown in Eq. 5.1. The idea that a protein may unfold or fold through a multistep process has in turn led to the "New View" of protein folding. Interestingly, much of the early data which led to this theory were from the milk protein, α-lactalbumin (Xie *et al.*, 1991; Farrell *et al.*, 2002b).

$$\text{Native} \rightleftharpoons \text{Intermediate} \rightleftharpoons \text{Unfolded} \quad (5.1)$$

According to the "New View," during folding, a protein chain may "sample" a significant amount of conformational space before settling into a selective energy minimum. Indeed, several false minima may lie quite close or even somewhat remotely removed from the true global minimum (Farrell *et al.*, 2006b). Such an intermediate area has been postulated to be the molten globule state as shown in Fig. 5.2 for the theoretical energy landscape of a "minimally frustrated" heteropolymer, as folding is viewed from top to bottom. The parameter, Q, is the global order parameter and represents a value of 1.0 for all interactions in the native state, and 0.0 for the completely unfolded protein. Reflection of the interactions between Q, E, and S results in a three-dimensional funnel. In the "New View" then the intermediate state of Eq. 5.1 is now defined as a multiplicity of states which includes not only the molten globule as defined in Fig. 5.2, but also a number of confomationally defined states which reside above the molten globule (MG) region (Uversky, 2002). These new states, working upward from the molten globule state of Fig. 5.2, have been previously defined as

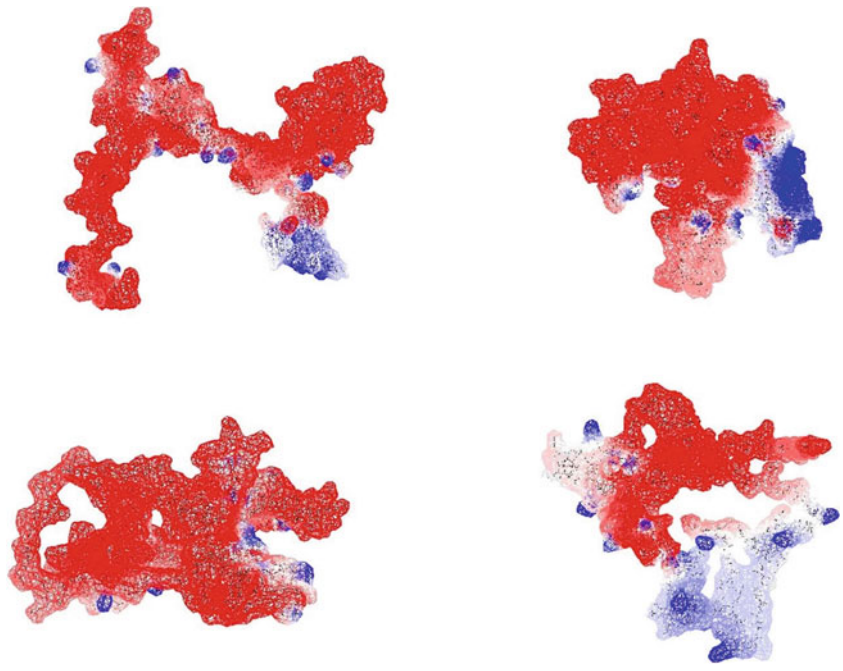

Fig. 5.1 Three-dimensional molecular models of the caseins obtained from sequence-based secondary structural predictions and aligned with spectroscopic data. *Top*: left α_{S1}-casein, right α_{S2}-casein; *bottom*: *left* β-casein and *right* κ-casein. The models display a pseudo-charged surface potential with *red* being negative, light being neutral and/or hydrophobic, and *blue* positive. These models represent a working view of casein structure and are subject to change as future experimentation progresses (Kumosinski *et al*., 1993a, b, 1994a; Farrell *et al*., 2009)

pre-molten globule (PMG) and natively unfolded (NU) based upon their physical and chemical properties (for a review, see Farrell *et al.*, 2006b). A different classification by means of biological function has also been attempted (Tompa, 2002; Tompa and Kalmar, 2010) and under the general heading of intrinsically unstructured protein (IUP), five functional definitions of these new states have been suggested. The question now arises, how can this "New View" be applied to casein structure?

The major hallmark of the IUP or NU proteins is that while they contain significant amounts of defined secondary structures, they do not fold and remain trapped in conformational energy states above that of the MG state depicted in Fig. 5.2, whereas native molten globules (which represent a subset of intrinsically disordered proteins) are trapped in the MG-like conformational energy states (see Fig. 5.2). This results from three general properties of these proteins:

1. They contain high contents of proline and glutamine leading to segments of polyproline II conformation (PPII), which produce extended structures with high hydration
2. They have a high net charge which prevents close approach of segments of the protein molecules
3. They usually have a low hydrophobicity which does not allow for hydrophobic collapse into a highly folded structure

With regard to the classification of the caseins in this scheme, they do have high amounts of proline and glutamine and have been shown to contain significant amounts of PPII (Farrell *et al.*, 2001; Syme *et al.*, 2002), they have high levels of hydration (Kumosinski *et al.*, 1988), and they have high net negative charges, resulting in part

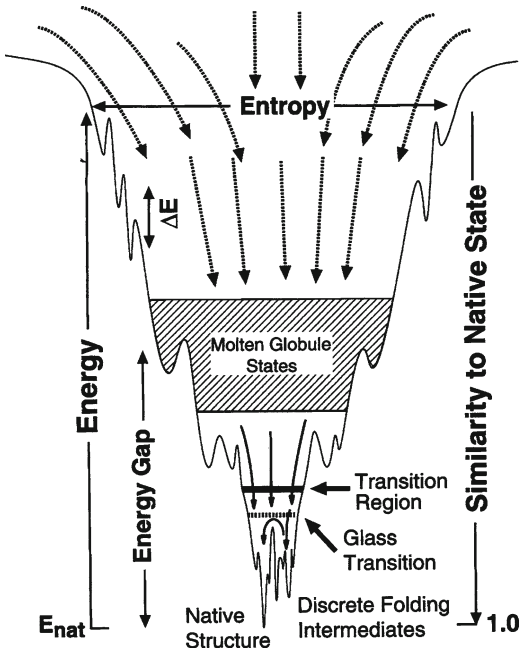

Fig. 5.2 A schematic representation for the energy landscape for a minimally frustrated heteropolymer during protein folding. E_{nat} is the minimum potential energy for the native state. From Onuchic *et al.* (2000); reprinted courtesy of Academic Press, San Diego, CA

from their phosphoserine residues (Farrell *et al.*, 2004). However, in contrast to many other IUP or NU proteins, they are highly hydrophobic, and it is this latter property which undoubtedly leads to their aforementioned propensity for self-association. Using these three criteria alone, it is somewhat difficult to assign the caseins to their place within this "New View" as shown in Fig. 5.2. However, Farrell *et al.* (2006c) have examined in detail each casein and its relationship to a variety of criteria developed by Uversky (2002) for classifications within these states. The overall results of these analyses are given in Table 5.1.

The hydropathy plots and PONDR® analyses are sequence-based binary predictors of disorder in proteins. These programs were used by Farrell *et al.* (2006c) to analyze 11 α_{S1}-CNs, 9 α_{S2}-CNs, 13 β-CNs, and 20 κ-CNs with known sequences. Using these methods, the α_{S1}- and α_{S2}-caseins are set apart from the β- and κ-caseins, primarily because of their high net negative charges. The β- and κ-caseins are predicted to be more compact but still well within the MG levels and all are clearly not ordered proteins.

For the NU, PMG, and MG states, the experimentally determined volume of a monomeric form of the protein is always much greater than the volume of a compact globular protein of the same chain length. Uversky (2002) has developed a series of equations to classify proteins by this method. Using these equations, κ-casein (with a reducing agent added) appears to be the most unfolded while the other three appear to be in the PMG state (Table 5.1). An additional volume-based calculation can be made for β- and κ-caseins. Because their polymeric states have been well described in terms of molecular weight and size, it is possible to calculate the volume of the β- and κ-casein monomers within their respective polymers and compare these using the Uversky (2002) equations. This analysis places both the β- and κ-casein monomers solidly within the MG state.

The 3D models as shown in Fig. 5.1 can be analyzed in terms of their size and shape as well, and the radius of gyration can be calculated from the monomer molecular volume. Applying the size/volume calculations to the 3D models yields a prediction for their states within Fig. 5.2 as shown in Table 5.1. Here, α_{S1}-casein is the most extended and predicted to be in the NU state, while α_{S2}-casein is more compact in the PMG state. However, the 3D models predict the MG state for the β- and κ-caseins, but these predictions most likely again represent the characteristics of the monomers within their respective polymers rather than an independent monomer state. Indeed, this was a prediction made during the original modeling of β-casein (Kumosinski *et al.*, 1993a).

Overall, then it may be concluded that α_{S1}-casein may occur in the NU state, α_{S2}-casein is more compact and falls into the PMG state, while both β- and κ-caseins as monomers within their polymers reside in the MG state. It is important to remember that none of these states is random coils and as noted above contain significant amounts of defined secondary structures, but do not, for various reasons, fold into compact globular proteins. Moreover, all of these predictions

Table 5.1 Comparison of classification "New View" schemes for α_{S1}-, α_{S2}-, β-, and κ-caseins.

Classification methodology				
Casein	Hydropathy plots[a]	Monomer size by GPC[b]	Monomer size from polymer data[c]	3D model size[d]
α_{S1}-	NU	PMG		NU
α_{S2}-	NU	PMG		PMG
β-	MG	PMG	MG	MG
κ-	MG	NU	MG	MG

NU natively folded, *PMG* pre-molten globule, *GPC* gel permeation chromatography
[a]Farrell *et al*. (2006c) for hydropathy and PONDR® analyses
[b]Pepper and Farrell (1982)
[c]Dynamic light scattering and sedimentation equilibrium (Farrell *et al*., 2006c)
[d]Kumosinski *et al*. (1994a, 1993a, b) for α_{S1}-, β-, and κ-CN; Farrell *et al.* (2009) for α_{S2}-CN

are for the caseins in the absence of added calcium or phosphate. The caseins, then, are solidly in the realm of these new protein classes and are a part of the rapidly emerging study area termed "unfoldomics" by Dunker *et al.* (2008). For the caseins, these open and more hydrophobic states lead to their intrinsic property of self-association. It could be said, then, that the native states of the α_{S1}-, α_{S2}-, β-, and κ-caseins are the states in which they exist when fully either immersed in the casein micelle or self-associated with themselves or other caseins where the hydrophobic regions of the caseins are intermingled. In the next section we will examine several examples of how predicted secondary structures for the casein monomers lead to their unique polymeric states.

5.3 Molecular Modeling of α_{S1}-Casein: Interactions and Support from Experimental Data

To exemplify how persistent secondary structures in the caseins may lead to aggregation and self-association, we will first focus on the primary calcium-binding protein of milk: α_{S1}-casein (Farrell *et al.*, 2004). The molecular model, presented for α_{S1}-casein in Fig. 5.1, shows two potential sites for self-association. The first is the C-terminal portion (residues 130–199) which may be responsible for pronounced salt-accelerated self-association (Schmidt and Payens, 1976); this region encompassing residues 140–190 is extremely hydrophobic in nature and is found between 10 and 7 o'clock in the 3D model of α_{S1}-casein (Fig. 5.1). A second site for aggregation may occur in the region 14–25, as the deletion of this region in the α_{S1}-casein A genetic variant leads to altered forms of self-association (Kumosinski and Farrell, 1991, 1994); this region also contains a chymosin-sensitive bond, cleavage of which leads to changes during cheese maturation (Tunick *et al.*, 1997). In contrast to the molecule as a whole, the N-terminal region (1–26 and 4 o'clock in the 3D model) is positively charged. Finally, Horne (1998) has suggested that these two segments represent surface-active regions of α_{S1}-casein.

5.3.1 Hydrophobic Dimers and Oligomers of α_{S1}-Casein

Schmidt and Payens (1976) and Schmidt (1982) summarized light scattering studies on variants of α_{S1}-casein under a variety of environmental conditions from which a stoichiometry of the α_{S1}-casein self-association is obtained at selected ionic strengths and at 20°C. From these results, it can be concluded that α_{S1}-casein undergoes a concentration-dependent, reversible, hydrophobically controlled association from monomer to dimer and then to tetramer, hexamer, and octamer,

Table 5.2 Weight-average molecular weights of selected caseins and mixtures by analytical ultracentrifugation at 37°C[a]

Casein or mixture	Wt.-Avg. molecular weight	Average polymeric size	Rotor speed
α_{S1}-Casein	56,000	Dimer	12,000
β-Casein	1,250,000	52-mer	3,000
RCM κ-casein	3,040,000	160-mer	3,000
1.5 α_{S1}-:1 RCM κ-	316,000	15-mer	3,000
4 α_{S1}-:1 RCM κ-	92,400	Tetramer	6,000
4β-:1 RCM κ-	1,010,000	43-mer	3,000
1β-:1α_{S1}-	213,000	Nonamer	3,000

[a]All data were obtained at 37°C, pH 6.75 in 25 mM disodium piperazine-N,N′-bis(2-ethane sulfonic acid) with 80 mM KCl to mimic milk salt conditions in the mammary gland in the absence of calcium; the rotor speeds were appropriate to the weight-average MW as previously described. *RCM* reduced and carboxymethylated. Modified from Farrell *et al.* (2006a)

and even higher levels if the ionic strength is increased to 0.6 M (Thurn *et al.*, 1987). These extended polymers are thought to be rodlike and may involve head-to-head (1–25) and tail-to-tail (140–190) condensations of the molecule shown in Fig. 5.1. Studies of the whole α_{S1}-casein molecule and its derivative, produced by carboxypeptidase activity (f1–197), which lacks Trp199, appeared to confirm these two areas as the primary interaction sites (Alaimo *et al.*, 1999a). However, recent work (Malin *et al.*, 2005) has shown that at 37°C and at physiological salt concentrations, the higher order polymers are broken down and that α_{S1}-casein is essentially a dimer under physiological conditions (Table 5.2). It has also been shown (Malin *et al.*, 2005) that this dimerization involves residues 136–158 of each monomer (10 o'clock in the α_{S1}-model of Fig. 5.1). These experiments demonstrate that for α_{S1}-casein, preformed structural elements surrounding Pro_{147} participate in the formation of dimers at conditions simulating physiological temperature, pH, and ionic strength, and neither the 1–25 region nor tryptophan 199 is involved.

To mimic this hydrophobic dimerization, Kumosinski *et al.* (1994a) constructed an energy-minimized dimer from the large-stranded β-sheets which occur at residues 136–158. The side chains are predominately hydrophobic, and limited hydrogen bonding of the sheet structure lends rigidity to this site. A dimer can be formed easily if two of these sheets are docked in an antiparallel fashion (Fig. 5.3), and this structure, following energy minimization, displays a stabilizing energy of −520 kcal mol^{-1} $residue^{-1}$ (Kumosinski *et al.*, 1994a).

5.3.2 Hydrophobic Dimers and Oligomers of α_{S1}-Casein Fragment f136–196

All of the above data appear to suggest that for α_{S1}-casein, preformed structural elements surrounding Pro_{147} participate in the formation of dimers. A better understanding of these interactions was brought about by investigating the hydrophobic portion of the protein, namely, the α_{S1}-casein (f136–196) peptide. Isolation of this sizable portion of the C-terminal half of the protein was achieved by cyanogen bromide cleavage and purification as described by Alaimo *et al.* (1999a, b). The global secondary structure of α_{S1}-casein (f136–196), as estimated by far-UV CD, is given in Table 5.3. At 27°C and pH 6.75 in a low ionic strength buffer, the results for α_{S1}-casein (f136–196) are consistent with the putative 3D structure in which all six proline residues are involved in turns. Here the peptide may be expected to be a dimer in equilibrium with its

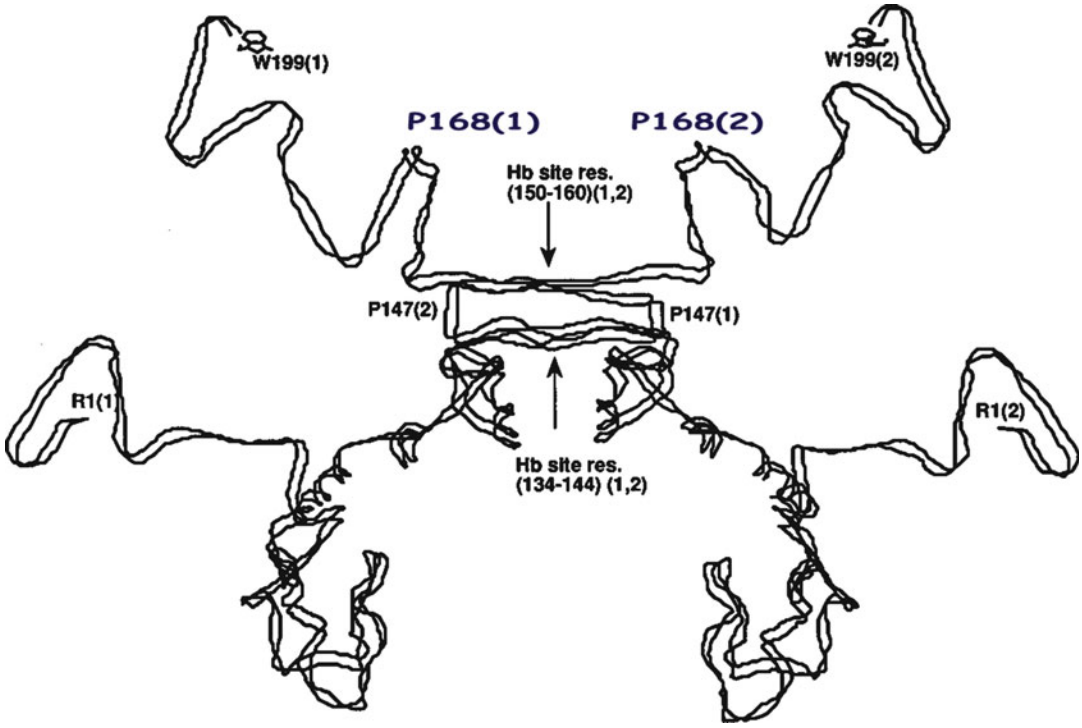

Fig. 5.3 Double ribbon structure of α_{S1}-casein dimer constructed by docking two large hydrophobic sheets in an antiparallel fashion interaction; sites are noted (*1*, *2* indicate molecules 1 and 2). *Hb* hydrophobic site of interaction, *W199* C-terminal tryptophan, *R1* N-terminal arginine, *P147* proline 147, *P168* proline 168 (Kumosinski *et al*., 1994a)

Table 5.3 Comparison of secondary structural estimates for the peptide α_{S1}-casein (f136–196) by three methods

Method	Temperature (°C)	β-Sheet (%)	Turns (%)	Unspecified (%)	α-Helix (%)
FTIR[a]	25	49 ± 3[b]	22 ± 1	23 ± 1	5 ± 2
CD[c]	10	64 ± 2	28 ± 1	5 ± 1	3 ± 1
	27	58 ± 1	31 ± 1	8 ± 1	3 ± 1
	50	49 ± 2	28 ± 1	18 ± 1	5 ± 1
	70	49 ± 2	29 ± 1	18 ± 1	3 ± 1
3D model	in vacuo	57	28[d]	15	0

[a]Average of three determinations in PIPES-KCl aqueous at pH 6.75 ionic strength 124 μM (Alaimo *et al.*, 1999b)
[b]For FTIR—includes 3_{10}-helix, bent strand, and extended β-sheet (Alaimo *et al.*, 1999b)
[c]For CD-average fits, one determination (six accumulations at each temperature) ionic strength 4 μM
[d]For molecular models—includes I-P-N-P-I loop at residues 182–186

component monomer and tyrosines 144, 146, and 154 served as reporter groups for this interaction. Global secondary structure was also estimated from the FTIR spectra of a 1.5% aqueous solution of α_{S1}-casein (f136–196) and at higher concentrations and ionic strengths where higher order aggregates occur at 25°C. The results of this analysis were in good agreement with the CD data (Table 5.3). However, as the peptide is heated, there is an increase in the calculated amount of unspecified (random-coil) structure and loss of β-sheet (Table 5.3). Malin *et al.* (2005) showed that in computer-generated molecular dynamics simulations of this peptide, the

sheet-turn-sheet motif directed by Pro_{147} was stable in an aqueous environment while others were not. For the peptide (f136–196) we propose that the turn region about Pro_{147} is the initial site of hydrophobic self-association (dimerization) at physiological conditions. Moreover, interactions centering on Pro_{168}, involving Trp_{164} as a reporter group, indicate that this region is involved in the higher order polymers found for the peptide (f136–196) at higher ionic strengths and at 25°C (Alaimo *et al.*, 1999a, b; Malin *et al.*, 2005). By extension, the regions seen at the top in Fig. 5.3 would represent sites for the formation of the higher order polymers found for the whole α_{S1}-casein molecule at high ionic strengths (Thurn *et al.*, 1987). Thus, the sheet-turn-sheet secondary structure motif is most likely the basis for the self-association behavior of this casein and as will be seen below for its vital interactions with other caseins.

5.4 Molecular Modeling of β-Casein: Interactions and Support from Experimental Data

β-Casein is the most hydrophobic casein and has the largest regions of high hydrophobicity (55–90 and 130–209) with a very acidic N-terminal region of 24 amino acids. The acidic N-terminal region is at the right top of the model in Fig. 5.1. There are two regions where plasmin readily cleaves the protein (at bonds 28–29 and 105–106/107–108, respectively; 3 and 4 o'clock in the β-casein model of Fig. 5.1). Cleavage at these sites by plasmin yields the fragments previously known as γ_1- and γ_2-caseins, respectively (Farrell *et al.*, 2004).

The self-association of this protein is (detergent) micelle-like (Qi *et al.*, 2005), and both ionic strength and temperature increase the quantity of polymer present (i.e., increased association constant) and the degree of association (n):

$$n\beta - CN \leftrightarrow \beta - CN_n \qquad (5.2)$$

The number of monomer proteins in these nearly spherical polymers ranges from about 15

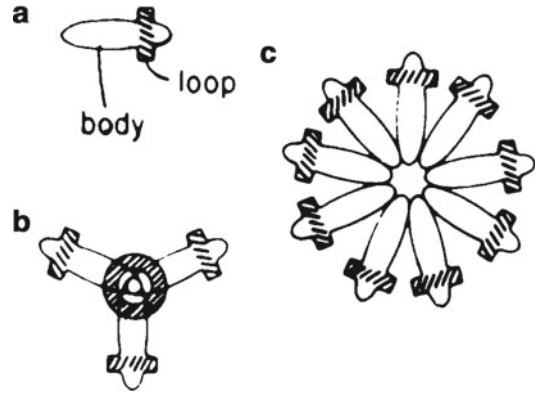

Fig. 5.4 Schematic representation of monomer and polymer models for α_{S1}- or β-caseins; (**a**) monomer, (**b**) tetramer, and (**c**) planar representation of a rosette-shaped spherical polymer. Waugh (1970), reprinted courtesy of Academic Press, San Diego, CA

at 0.1 M ionic strength, pH 7, and 20°C to about 52 at 0.110 M ionic strength, pH 6.7, and 37°C (Table 5.2). Perhaps the best representation of this process was given by Waugh (1970) originally for α_{S1}-casein, but by extension to β-casein. As shown in Fig. 5.4, the phosphopeptide is represented as a ring and the remainder of the molecule as a torpedo-like structure. Overall, the geometry of the β-casein polymers as given in Fig. 5.4 is still in accord with data elucidated over the past 40 years. However, as noted above, the intrinsic volume of an individual β-casein within the polymer is significantly lower than the monomer, so Waugh's monomer still represents the monomer within the polymer in a molten globule-like state exemplified by the 3D model for β-casein (Fig. 5.1). β-Casein has one site which is particularly sensitive to chymosin (189–190/192–193) found at about 7 o'clock in the β-model of Fig. 5.1. As shown by Qi *et al.* (2005) loss of the C-terminal peptide (193–209) by chymosin cleavage substantially reduces but does not eliminate the association-dissociation equilibrium. At pH 6.75, 37°C, and ionic strength 50 mM, the weight-average MW drops from 495,000 to 90,222 Da (Table 5.4). The latter represents a tetramer as the monomer molecular weight for f1–192 of β-casein is 22160 Da (Qi *et al.*, 2005). Thus, the peptide f193–209 is a requisite for the normal self-association of β-casein.

Table 5.4 Weight-average molecular weights, apparent association constants of selected caseins and mixtures by analytical ultracentrifugation at 37°C

	Analytical ultracentrifuge				
	MW[b]	Protomer MW[c]	k_a[d] (L g^{-1})	n_a	Rotor speed (rpm)
α_{S1}-Casein	56,000	56,000	1.09×10^{-1}	4	12,000
β-Casein[f]	495,000	24,000	4.38×10^{14}	29	6,000
β-Casein[f] (f 1–192)	90,200	22,000	4.68×10^{-3}	18	10,000
RCM κ-casein	4,140,000	505,000	1.35×10^{11}	16	3,000
4 α_{S1}-:1 RCM κ-	92,400	69,800	nd[e]	nd[e]	6,000
4β-:1 RCM κ-	1,010,000	711,000	5.82×10^{-6}	4	3,000
1 β-:1α_{S1}-	213,000	100,000	7.36×10^{-3}	4	6,000

[a]All data except where noted were obtained at 37°C, pH 6.75 in 25 mM disodium piperazine-N,N′-bis(2-ethane sulfonic acid) with 80 mM KCl to mimic milk salt conditions in the mammary gland in the absence of calcium; the rotor speeds were appropriate to the weight-average MW as previously described. *RCM* reduced and carboxymethylated. Modified from Farrell *et al.* (2006a)

[b]Weight-average MW in Da, three determinations ± 5%

[c]The protomer is the lowest molecular weight found at the top of the cell at equilibrium; it represents the kinetically active species which participates in association reactions as described in Equation (5.2)

[d]k_a and n_a as described in Equation (5.2)

[e]These quantities were not determined

[f]These β-casein experiments were conducted at a lower salt concentration (I = 50 mM) to accentuate the differences between the intact molecule and f1–192 (Qi *et al.*, 2005)

The question now as stated above is, does this segment contain any persistent periodic secondary structures which contribute to this self-association?

CD and FTIR spectral analysis indicates that at 6°C, β-casein has a relatively low level of α-helix (~15%), an intermediate level of turn-like structure (~29%), and a similar level of extended or β-sheet (~30%) in dilute, low ionic strength, neutral solutions. However, there are significant increases in (CD) ellipticity at 220 nm when the solution temperature is increased from 6 to 37°C. β-Casein is predicted to contain a significant amount (30%) of polyproline II structure, but these structures have been shown to occur at about 20% in β-casein by Raman spectroscopy, FTIR and CD (Farrell *et al.*, 2001; Syme *et al.*, 2002). Thus, it is possible that increases in both β-strand and polyproline II structure are responsible for this temperature-dependent structural change.

It has been suggested for a growing number of proteins (Qi *et al.*, 2005) that reactions at their C-terminal regions are important for their biological function. A notable example is tailspike protein produced by phage 22 which infects *Salmonella* spp. The tailspike protein through its C-terminal region binds to the surface of the bacterial cells. As seen in its crystal structure (protein data bank # 1TYW; Steinbacher *et al.*, 1994), this region contains a section of the sheet-turn-sheet motif (residues 632–666) as shown in Fig. 5.5 (top). Interestingly, residues 636–655 of the tailspike protein have a 64% homology with f193–209 of β-casein. Using the crystal structure coordinates of this segment of the tailspike protein, it is possible to construct a homologous molecular model for f193–209 of β-casein. To do this the peptides were aligned as described by Qi *et al.* (2005); four residues found in the center of a β-sheet in tailspike protein but not in β-casein were deleted as were seven residues at the C-terminal end. The selection was then submitted to the ExPASy web site for the production of a homologous model, as described by Farrell *et al.* (2009) for α_{S2}-casein. A representation of the homologous structure is shown in Fig. 5.5 (bottom) and it retains the sheet-turn-sheet motif of

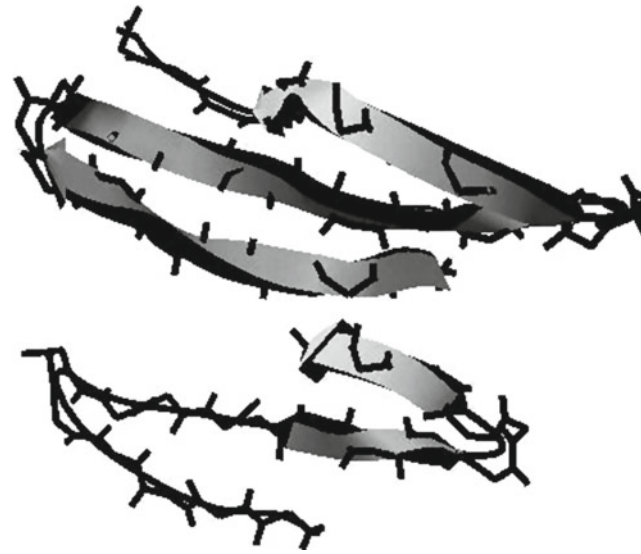

Fig. 5.5 On the top is the C-terminal portion of the tailspike protein (phage22) residues 657–688 with its C-terminal at the top and the arrowheads represent the direction of travel of the chain from N- to C-terminal with a stable sheet-turn-sheet motif (protein data bank # 1TYW, Steinbacher *et al.*, 1994). On the bottom is the homologous model for the C-terminal peptide of β-casein f193–209 as generated from the tailspike peptide. This may also represent a model for casecidins 15 and 17 (Birkemo *et al.*, 2009)

the original model, but with weaker hydrogen bonding. This model may be of use in that the sequence corresponds to casecidins 15 and 17 (Birkemo *et al.*, 2009). These two peptides have antimicrobial activity toward *E. coli* and represent f192–209 and f192–207 of β-casein. It is interesting to speculate that the antimicrobial action of the peptides is mechanistically and structurally related to the action of the phage22 protein on *Salmonella*.

Next, the coordinates obtained for the β-casein peptide f192–209 were transferred to the whole β-casein model of Fig. 5.1 and following the regimen of Farrell *et al.* (2009), this leads to the extension in space of the original C-terminal section of the molecule as now given for β-casein in Fig. 5.6. Here the extended C-terminal region would be free to interact with other β-caseins and to self-associate into the large polymers shown in Fig. 5.4 for Waugh's model. In the sleek representation in Fig. 5.4, no such tails are shown and a void is depicted at the center of the cross section. When Kumosinski *et al.* (1993a) constructed polymers of β-casein from the 3D model of Fig. 5.1, similar voids occurred. The void in Fig. 5.4 can be calculated to be about 22,000 Å^3. Measuring the volume of the extended tail as 588 Å^3, about 37 tails could be placed into this cavity area. It should be remembered that chymosin cleavage of this sheet-turn-sheet area dramatically reduces β-casein self-association. So as noted above, these residues may well coalesce to anchor the β-casein monomers to their spherical polymer. This provides another example of how a sheet-turn-sheet motif in a casein may lead to its characteristic mode of self-association.

5.5 Molecular Modeling of κ-Casein: Interactions and Support from Experimental Data

κ-Casein, which constitutes 10–12% of whole casein, plays a crucial role in stabilizing the casein micelles in milk and, after enzymatic cleavage, destabilizing the colloidal casein system (Farrell *et al.*, 2004). The enzymatic cleavage that brings about this transformation is important for the nutrition of the suckling young and for the production of many cheese varieties. This is achieved by the molecule having two distinctly different domains. As seen at the bottom

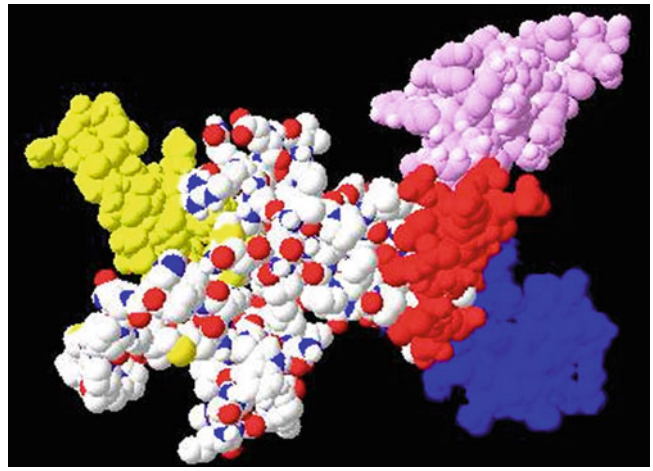

Fig. 5.6 The original model of β-casein as shown in Fig. 5.1 is rotated 90° toward the viewer and has had its psi, phi, and omega angles of residues 192–209 changed to conform to those of the peptide of Fig. 5.5. The *red* color represents the N-terminal phosphopeptide region, the *pink* a region of lower net charge containing the first plasmin cleavage site (residues 28–29), the *blue* region indicates the second plasmin cleavage site (residues 105–107), the white colored portion represents the bulk of the hydrophobic body, and the *yellow* represents the C-terminal section (f193–209) modeled after the tailspike peptide and extended in space for potential polymerization reactions

portion of the κ-model in Fig. 5.1, the N-terminal domain (residues 1–95) carries a net positive charge, is very hydrophobic, and interacts strongly with the other casein molecules. The C-terminal domain (residues 113–169) carries a net negative charge and contains a preponderance of polar residues (top portion of the κ-model in Fig. 5.1). These two domains are joined by a peptide (residues 96–112, 10 o'clock in the κ-casein model of Fig. 5.1) that carries a net positive charge, is predicted to be a β-strand, and is generally well conserved in most species (Palmer *et al.*, 2010). This region contains a motif that is readily recognized by chymosin and is rapidly and specifically cleaved to give the two domains noted above. The peptide f106–169 is called caseinomacropeptide (CMP) or glycomacropeptide (GMP) because about half of the κ-casein molecules are posttranslationally glycosylated and phosphorylated (Farrell *et al.*, 2004). Additionally, all of these sites are surface orientated in the κ-casein model (Fig. 5.1).

The original model of κ-casein (Fig. 5.1) contained a segment of alpha helix for the chymosin site; most recent work has clearly demonstrated that a β-sheet structure is required for the chymosin active site (Palmer *et al.*, 2010). Accordingly, the κ-casein wire model shown in Fig. 5.7 has a seven-residue segment of β-sheet in this region, and Phe_{105} and Met_{106} are clearly visible. In the altered model the proline residues that precede and follow this segment of β-sheet put a significant strain on the region, and hydrolysis results in increased disorder (entropy) in silico.

As purified from milk, κ-casein occurs as a series of intermolecular disulfide-bonded aggregates (Groves *et al.*, 1992). Farrell *et al.* (1996) produced a 3D model of a κ-casein octamer composed of two disulfide-bonded tetramers to explain the overall properties of κ-casein as purified from milk. This octamer is shown in Fig. 5.8 (left) and displayed with the same pseudocharges of Fig. 5.1; it is deep red (negative). It is generally accepted that the "hairy" GMP (Horne, 1998, 2006) provides steric hindrance and prevents casein micelles from coalescing in normal milk. When the GMP is removed by chymosin, it is thought to bring about

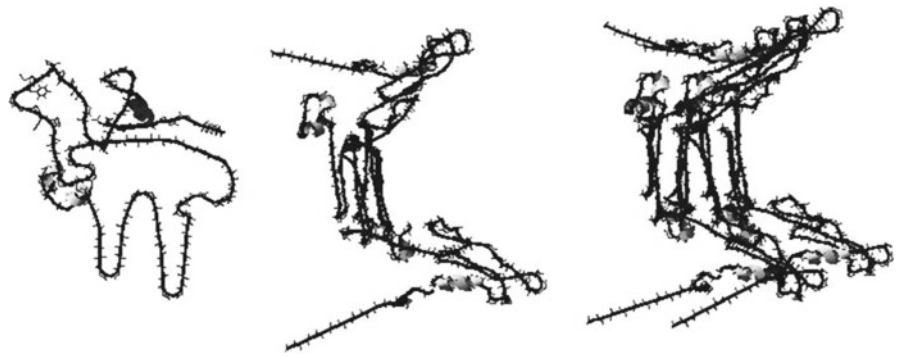

Fig. 5.7 The original monomer model of κ-casein as shown in Fig. 5.1 is modified here to show a segment of β-sheet for the chymosin cleavage site at 10 o'clock (*left*) and the phenylalanine-methionine residues are in the center of the β-sheet. In this model the sulfhydryl groups are blocked. The molecular model thus allows for the formation of dimers (*center*) and tetramers (*right*). The further propagation of the polymeric structures from the reduced monomer is responsible for the formation of the high molecular weight amyloid structures (Farrell *et al.*, 2003b)

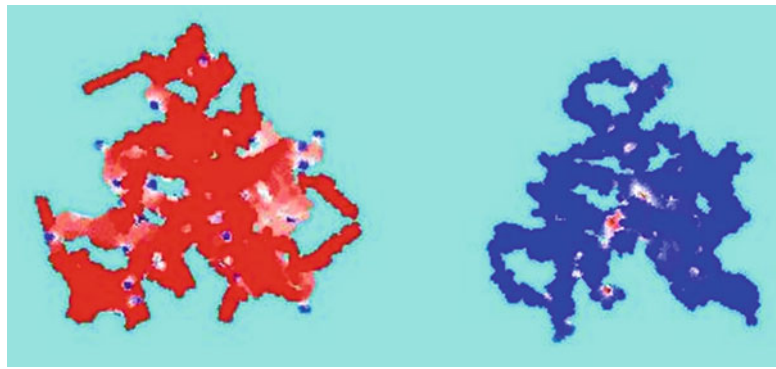

Fig. 5.8 Molecular model for a κ-casein octamer composed of two disulfide-bonded tetramers. This model can be thought of as a representation of a poly κ-casein area on the surface of a micelle. The model on the *left* represents intact κ-casein octamer and is colored red representing a high negative electrostatic potential. When the negatively charged GMP is removed in silico, there is a complete charge reversal (*right*) where *blue* represents positive charge

aggregation of the casein micelles in milk because of loss of this hindrance. However, as seen in the 3D model for an octamer of κ-casein, when the negatively charged GMP is removed in silico, there is a complete charge reversal in Fig. 5.8 (right) where blue represents positive charge. Thus, the creation of surface positive charge may enhance the aggregation of casein micelles following chymosin action through charge–charge interactions with the abundance of surface negative charges on other caseins.

Circular dichroism (CD) and FTIR spectral analysis indicates that for κ-casein there is a relatively low level of α-helix (15%), an intermediate level of turn-like structure (~25%), and a higher level of extended or β-sheet (~30%). The above molecular models for κ-casein are in accord with these measurements. In addition, all of these structures appear to be thermostable, as shown in Table 5.5 (Farrell *et al.*, 2003b).

As noted above, κ-casein exists as a series of intermolecular disulfide-bonded aggregates, and

Table 5.5 Comparison of secondary structural estimates for κ-casein by three methods

Method	Temperature (°C)	β-sheet (%)	Turns (%)	Unspecified (%)	α-helix (%)
FTIR[a]	25	35 ± 3[b]	25 ± 2	23 ± 4	17 ± 2
CD[c]	25	40 ± 1	26 ± 1	24 ± 1	9 ± 1
	10	36 ± 2	28 ± 1	24 ± 1	12 ± 1
	50	36 ± 2	27 ± 1	24 ± 1	14 ± 1
	70	36 ± 2	24 ± 1	20 ± 1	19 ± 1
3D model	in vacuo	30	32	30	10

[a]Average of three determinations in PIPES-KCl aqueous at pH 6.75 (Farrell *et al.*, 1996)
[b]For FTIR—includes 3_{10}-helix, bent strand, and extended β-sheet (Farrell *et al.*, 1996)
[c]For CD-average fits, one determination (six accumulations at each temperature) (Farrell *et al.*,2003b)

these disulfide bonds may be formed after the casein micelles have been assembled in the epithelial cells (Farrell *et al*., 2006a). These disulfide aggregates range from dimers to octamers and above (Groves *et al.*, 1992). However, about 5% of the cysteine residues in total are not disulfide-bonded and may react with other proteins or participate in oxidation reduction reactions, e.g., κ-casein aggregation with heat (Groves *et al.*, 1998) or formation of complexes with β-lactoglobulin in heated products (Haque *et al.*, 1987; Douglas *et al.*, 1981). As a reduced isolated protein, at 20°C κ-casein self-associates in a similar manner to β-casein to a 600,000 Da molecular weight polymer. However, the reduced carboxymethylated (RCM) protein at 37°C forms amyloid bodies which were first discovered in this casein (Farrell *et al.*, 2003b). These amyloids have a soluble molecular weight of over 3×10^6 Da (Table 5.2). The β-sheet structures at 6 o'clock of κ-casein monomer in Figs. 5.1 and 5.7 are thought to promote amyloid formation. In both CD and FTIR studies there is little change in the overall content of secondary structure during fibril formation. This type of sheet-turn-sheet interaction was predicted for the model as shown in Fig. 5.7 (Farrell *et al.*, 2003b). In subsequent studies, X-ray diffraction data were found to be in accord with the model presented in Fig. 5.7 (Thorn *et al.*, 2005).

Thus for κ-casein, like β- and α_{S1}-caseins, a defined sheet-turn-sheet secondary structure leads to protein–protein interactions. Here, however, instead of self-association this polymerization is more properly classified as an aggregation. Another example in κ-casein is the β-sheet structure which is surrounded by proline turns and which contains the chymosin-sensitive bond. The accurate hydrolysis of this bond initiates the first step in the digestive process and again represents how the defined persistent secondary structure of a casein leads to ultimate biological function.

5.6 Molecular Modeling of α_{S2}-Casein: Interactions and Support from Experimental Data

α_{S2}-Casein is the least hydrophobic and the most highly and variably phosphorylated of the caseins. There are three phosphopeptide regions (5–18, 49–68, and 126–145) in the casein sequence (left side of the model of α_{S2}-casein in Fig. 5.1) and a large central hydrophobic region (90–120) with very little charge (region of α_{S2}- protruding from the center of the model to 6 o'clock in Fig. 5.1). There is a second large hydrophobic region (160–207), but it has a number of positively charged residues, which represent the highest positively charged area for any casein (Fig. 5.1). In milk, the majority (~90%) of the protein occurs with an internal disulfide bond between cysteine residues 36 and 40 forming a small loop in the structure. In addition a small proportion of this protein exists as a disulfide-bonded dimer as well as polymers with κ-casein (Farrell *et al.*, 2009).

Circular dichroism (CD) and FTIR spectral analysis indicates that for α_{S2}-casein there is a relatively high level of α-helix (30–40%), an intermediate level of turn-like structure (~20%), and a similar level of extended or β-sheet (~20%). Prediction methods indicate that likely positions for helix formation are near both the acidic N-terminal and the basic C-terminal regions (Hoagland *et al.*, 2001). This protein is readily hydrolyzed by plasmin and trypsin at a number of sites primarily in the afore noted C-terminal region, so that at neutral pH these positively charged residues are primarily at the surface and could actively participate in the binding of inorganic phosphate. In addition, on proteolysis, this area gives rise to a number of biologically active peptides with defined structures (Farrell *et al.*, 2009).

Association studies at 20°C show that monomeric α_{S2}-casein behaves in a very similar way to α_{S1}-casein except that there is a maximum association at salt concentrations above 0.2 M (Snoeren *et al.*, 1980). However, at 37°C this protein has been found to form elongated amyloid structures similar to those formed by κ-casein (Thorn *et al.*, 2008). These interactions are thought to occur through associations of the central hydrophobic core (residues 90–120) noted above. This section of α_{S2}-casein has a strong homology with the portion of κ-casein responsible for amyloid production. Again, for this protein, amyloid formation occurs through sheet-turn-sheet structural motifs. This represents another example of aggregations driven by defined structural states.

5.7 Molecular Modeling of Casein–Casein: Interactions and Support from Experimental Data

In contrast to the number of excellent studies on the interactions of individual casein species in solution, there have been only a few studies of mixed associations involving primarily binary mixtures. Historically, such studies have shown that, in the absence of polyvalent cations and at 37°C, α_{S1}- and κ-caseins associate most strongly (Waugh, 1970; Farrell *et al.*, 2006a). A summary of changes in weight-average MW for selected casein–casein interactions is given in Table 5.2.

5.7.1 Mixed Associations of the Caseins

More in-depth analyses of the above interactions were attempted and the results are given in Table 5.4. With regard to Table 5.4, it is important to understand the meaning of the term "protomer." In the independent self-association reactions of α_{S1}-casein at low ionic strength and for β-caseins (Alaimo *et al.*, 1999a; Farrell *et al.*, 2001; Qi *et al.*, 2005), the protomer is the lowest molecular weight species found near the top of the equilibrium cell and usually represents the species which then goes on to yield polymers. For β-casein (Eq. 5.2) and its chymosin fragment, the protomers are the monomers which is also true for α_{S1}-casein at low ionic strength and at 25°C (Alaimo *et al.*, 1999a). However, as seen in Table 5.4, α_{S1}-casein at 37°C and physiological ionic strength is essentially a dimer and there is little self-association beyond this ($k_a = 10^{-1}$), so that the protomer approximates the weight-average MW (Malin *et al.*, 2005); no monomer is apparent. For RCM κ-casein at 37°C, the protomer leading to amyloids is 505,000 Da; again this is the smallest species present in the experiment. In the case of the β-casein-RCM κ-casein mixtures, the protomer and weight-average MW appear to be large and similar, but considerably smaller than κ-casein alone; also the association constants are substantially reduced from $>10^{11}$ to 10^{-6}. It should be noted that bovine RCM κ- and human β-caseins form similar complexes and go on to form casein micelles, in vitro, with added calcium and phosphate (Sood *et al.*, 2006).

For 1:1 α_{S1}–β-casein mixtures, the high molecular weight polymers of β-casein are reduced in size; the association constant for β-casein is reduced from 10^{14} to 10^{-3} at 6,000 rpm in Table 5.4. The equations predict a protomer of 100,000 Da, with a weight-average MW of 213,000 Da. These latter values are considerably smaller than those found at 3,000 rpm; studies at

9,000 and 12,000 rpm gave still smaller numbers. Such behavior indicates a case of pressure-dependent aggregation which can be a hallmark of highly hydrophobically driven interactions (Hummer *et al.*, 1998; Farrell *et al.*, 2002c); extrapolation to atmospheric pressure yields a value of 247,000 for the weight-average particle of the α_{S1}–β-casein mixture, possibly a decamer. Here α_{S1}-casein acts as a molecular detergent to reduce greatly the size of the β-casein polymers reducing the weight-average MW from over one million to the extrapolated value of 247,000 Da (see Table 5.2 for comparable ionic strengths). Using the latter molecular weight and a Stokes radius from Pepper and Farrell (1982), the molecular volume reflects a mixture of the NU volume of α_{S1}-casein and the MG volume of β-casein. This represents a departure from the model of Waugh (1970) who believed that in the mixed polymers of α_{S1}- and β-casein shown in Fig. 5.4, the individual α_{S1}- and β-caseins were nearly identical in shape and interchangeable without an effect on particle size.

Early studies on the weight ratios for the formation of α_{S1}-:κ- particles gave estimates from 1:1 to 10:1; later values centered on 4:1 (Waugh, 1970; Kumosinski *et al.*, 1994b). These earlier experiments were conducted with whole κ-casein which we now know represents a mixture of disulfide-bonded aggregates. The clearest example of this problem was demonstrated by Pepper (1972) using gel chromatography. In the latter experiments, α_{S1}-casein clearly reduced the size of the κ-casein aggregates, but the interaction peaks demonstrated large polydispersity. The weight-average MW of RCM κ- is reduced by α_{S1}-casein at a 1.5:1 ratio to 316,000 Da (Table 5.2). Thorn *et al.* (2005) showed that amyloid formation was not repressed at this ratio and required a weight ratio of nearly 4:1 for nearly complete repression. The inability of α_{S1}-casein at low weight ratios to depress fibril formation argues against these 1:1 complexes as a driving force for micelle formation as previously suggested (Waugh, 1970). For an α_{S1}-:κ- ratio of 4:1, where amyloid production is repressed (Thorn *et al.*, 2005), the molecular weight is reduced (Table 5.2). The particle does not appear to act as a single species, while a protomer molecular weight of 69,800 Da (possibly representing two α_{S1}-+ one κ-) was found, fitting to the progressive association equations yielded poor results with n and k not determined accurately (Table 5.4); however, a small amount of high molecular weight amyloid could skew the data. As was the case for α_{S1}-β-casein interaction, increasing the speed of the rotor greatly reduced the weight-average MW indicating pressure-dependent hydrophobic interactions. Extrapolation to atmospheric pressure yielded a weight-average MW of 117,000 Da, which approximates a 4:1 ratio. So, using amyloid repression and the above data as a guideline, overall the 4:1 ratio appears best but perhaps not unique. Interestingly, once β-casein is added to these mixtures and the concentrations are increased to those found in milk, more uniform complexes occur, as a result of up to 6–8 association reactions, and sodium caseinate-like particles are found (Slattery and Evard, 1973; Schmidt and Payens, 1976).

In the "New View" of protein structure noted above, α_{S1}-casein can be considered to be a natively unfolded (NU) "assembler" in that it is able to break down aggregates of β- and κ-casein and lead to successful transit through the mammary secretory system prior to the addition of calcium in the Golgi apparatus. Indeed, its absence in the caprine homozygous null α_{S1}-allele (Chanat *et al.*, 1999) leads to the accumulation of large protein particles in the endoplasmic reticulum resulting in reduced secretion. Although, as noted above β-casein can repress κ-casein amyloid formation, β-casein knockout genes in rats do not appreciably alter secretion. β-Casein appears to be phosphorylated and to enter into the Golgi secretory granules at a later time than α_{S1}-casein. This makes a strong case for α_{S1}-casein as the primary force in casein micelle secretion (Chanat *et al.*, 1999; Le Parc *et al.*, 2010). Recently, it has been shown that the amyloid bodies formed by α_{S2}-casein can be disassembled by α_{S1}-casein but not by β-casein (Thorn *et al.*, 2008). It is also interesting that in vitro caseins can aid in enzyme folding acting in a limited way as chaperones (Farrell *et al.*, 2006a).

5.7.2 Sodium Caseinate

Sodium caseinate (whole casein in the absence of divalent cations) is an excellent ingredient and finds many applications in food processing. This product can have somewhat varying properties depending on its method of preparation (Douglas *et al.*, 1981). Heating sodium caseinate at 140°C brings about a conversion to larger aggregates, due only partly to the free sulfhydryl groups of κ-casein (Chu *et al.*, 1995). Properties, such as viscosity, of casein solutions are concentration-dependent and a 15–20% casein solution is very viscous because of the associations of the molecules with one another. A number of emulsion and foaming studies have been done using either various commercial whole proteins, e.g., total milk protein (TMP) or milk protein concentrate (MPC) or whole casein materials. However, it must be stressed that at the core of the predictable functionality of the caseins lies the strong, rather selective, protein–protein interactions discussed above.

Laboratory preparations of sodium caseinate through mixed associations of the individual caseins discussed above bring about the formation of a rather stable polymer with an average diameter of 18 nm and a MW of about 280,000 Da at room temperature. As viewed by electron microscopy, these particles appear to be rather uniform in shape and size (Kumosinski *et al.*, 1996). However, at 37°C, these polymers can dissociate into monomers and smaller polymers at concentrations below 1% (Pepper and Farrell, 1982). An apparent dissociation constant can be calculated to be 6.2 mg/mL from Pepper and Farrell (1982). Thus, for the casein found in the endoplasmic reticulum, prior to any addition of calcium and phosphate (Chanat *et al.*, 1999), in Golgi apparatus during phosphorylation (Farrell *et al.*, 2006a) and in milk (about 27 mg/mL), strong casein-casein associations are favored, as the concentrations are threefold greater than the dissociation constant. In all studies of lactating mammary tissue, sodium casein-like particles (putative submicelles) are abundant in electron micrographs of the mammary secretory process (Farrell *et al.*, 2006a; Chanat *et al.*, 1999; Schmidt, 1982).

5.7.3 3D Models for Sodium Caseinate (Particles in Solution)

Interpretations of all of the physical chemical data collected on sodium caseinate lead to a pointillistic or smeared view of the particles. To develop further a molecular basis for structure-function relationships of the sodium caseinate system, an energy-minimized 3D structure of a putative casein submicelle (sodium caseinate particle) was constructed consisting of one κ-casein, four α_{S1}-casein, and four β-casein molecules (Kumosinski *et al.*, 1994b). The models for the individual caseins were from the 3D structures depicted in Fig. 5.1 and the primary interactions used were those given above: two hydrophobically driven dimers of α_{S1}-casein interact with the β-sheet "legs" of κ-casein, thus preventing amyloid formation (a 4:1 ratio); secondarily, two dimers of β-casein, held together by their C-terminal peptides, interact at a later time with the κ- and α_{S1}- complexes. The construction of the model was described in detail in the previous version of this chapter (Farrell *et al.*, 2003a) and will not be repeated here. This modeling yielded two energy-minimized 3D structures for the putative casein submicelle and the more favored one is shown in Fig. 5.9. This refined κ-casein-based submicellar structure was tested by generating theoretical small-angle X-ray scattering (SAXS) curves and comparing them with experimental data (Kumosinski *et al.*, 1994c). Excellent agreement between experimental and theoretical curves was found. The global shapes and sizes of these putative submicellar structures were further tested by comparison of their computer-generated van der Waals dot structures with actual transmission electron micrographs (EM) of reduced and carboxymethylated whole casein (Kumosinski *et al.*, 1996). These comparisons, as shown in Fig. 5.10, demonstrate quite good agreement between the models and the EM. In actuality then the precise molecular coordinates of the 3D models can yield back the pointillistic views from which we started; the work has gone full cycle.

Very little data are available concerning the role of α_{S2}-casein in sodium caseinate structure

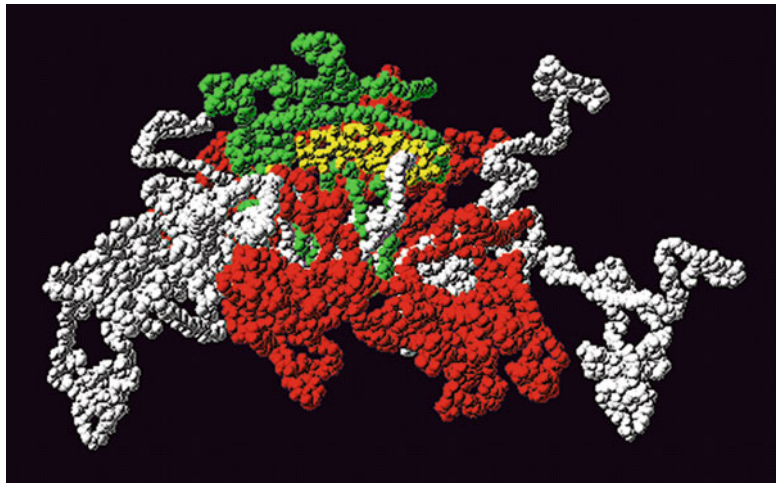

Fig. 5.9 Energy-minimized casein asymmetric submicelle structure, i.e., one κ-casein, two α_{S1}-casein dimers, and two β-casein symmetric dimers. Space-filled backbones without side chains; κ-casein B in *green*, α_{S1}-casein in white, β-casein A^2 in *red*, and bound water in *yellow* (Kumosinski *et al*., 1994b)

and formation. However, the α_{S2}-casein molecule has several similarities with κ-casein that are worth noting. First, residues 77–118 have a strong homology with residues 42–84 of κ-casein and both proteins form amyloid tangles on heating; the κ-casein amyloid centers on residues 26–85, while amyloid fragments of α_{S2}-casein found in mammary gland begin with residue 81 (Niewold *et al.*, 1999). Secondly, in their monomer states (Fig. 5.1) both have central hydrophobic cores apparently available for hydrophobic interactions which contain the latter noted regions. Additionally, α_{S1}-casein inhibits amyloid formation at a ratio of 4:1 for κ-casein, and a ratio of 2:1 for α_{S2}-casein. Hoagland *et al.* (2001), prior to the model for α_{S2}-casein, speculated that on the basis of structural similarity, perhaps α_{S2}-casein could compete with κ-casein as a primary interactant in the sodium caseinate particle. The new α_{S2}-casein model can do precisely that as demonstrated in Fig. 5.11 for the asymmetric submicelle model; note the area of positive charge on the surface of this model. This model has not been rigorously tested in the same fashion as the κ-casein-based submicelle model (Farrell *et al.*, 2003a) and serves only to suggest that α_{S2}-casein could play a more important role in casein structure than previously thought. More research is necessary to prove this speculation but almost all data now point to κ-casein as occurring as disulfide-bonded polymers and so some particles in the rather uniform fields seen for sodium caseinate may be κ-casein-rich while others may contain no κ-casein. Such a theoretical particle is shown in Fig. 5.11. That sodium caseinate might contain particles of different composition was suggested by Slattery and Evard (1973) based upon mixed associations of the caseins in sedimentation velocity studies at elevated ionic strengths. Interestingly, the above model offers a reply to the critical question (Horne, 2006) as to how to account for submicelles of varying composition.

If all of the caseins contain persistent structures, such as those seen above, then can they also be open and flexible, as their physical data suggest?

5.7.4 FTIR Studies of Sodium Caseinate (Casein Submicelles)

Curley *et al.* (1998) studied the effects of Ca^{2+} and Na^+ or K^+ on the FTIR spectra of sodium

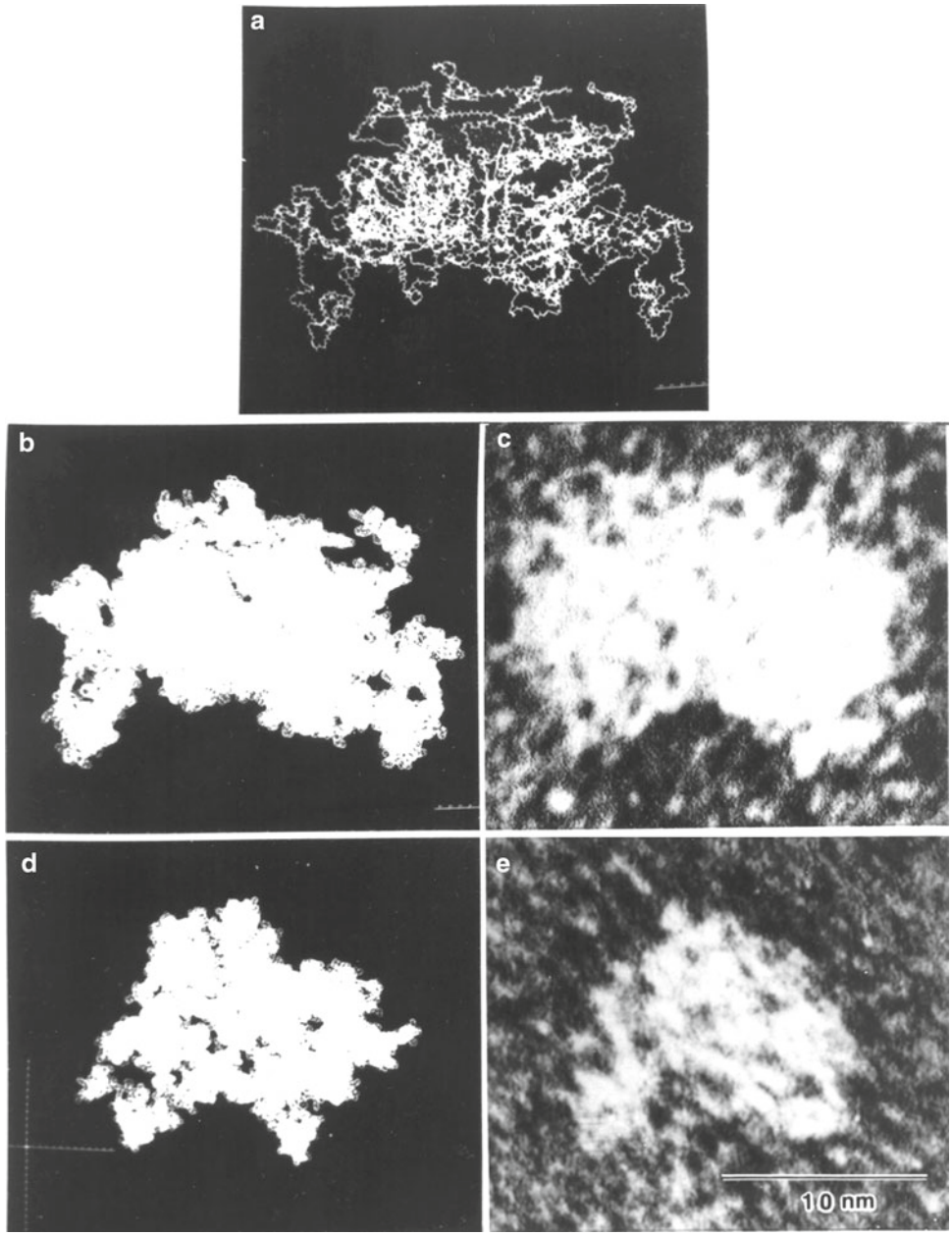

Fig. 5.10 Comparison of matched shape and dimensions of the asymmetric submicelle 3D model with photographically enlarged image enhanced representations of submicelles (TEM bar = 10 nm, molecular model = 5 nm). (**a**) Backbone structure for asymmetric model, (**b**) van der Waals dot surface model, (**c**) enlargement of image-enhanced micrograph, (**d**) van der Waals of (**a**) rotated 90° about *y*-axis, and (**e**) enlargement of image-enhanced representation of the submicelle particle (Kumosinski *et al*., 1996)

caseinate. They concluded that electrostatic binding of Ca^{2+} to casein resulted in a redistribution of the protein components of the infrared spectra. Addition of Ca^{2+} in salt solutions of K^+ and Na^+ led to apparent decreases in large loop or helical structures at 37°C with concomitant increases in the percentage of structures having greater bond energy, such as turns and extended helical

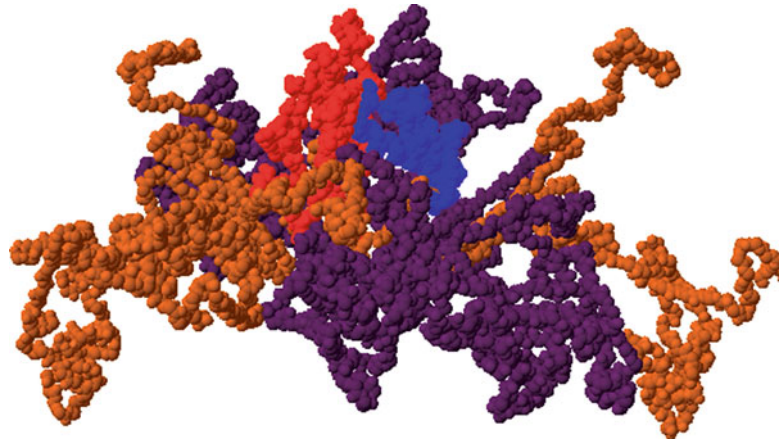

Fig. 5.11 Energy-minimized casein asymmetric submicelle structure; here the one κ-casein is replaced by one α_{S2}-casein. The model shows backbone atoms only in *brown* for two α_{S1}-casein dimers and *purple* for two β-casein dimers. The α_{S2}-casein is in red except for the basic C-terminal which is in *blue*

structures. The effects of Ca^{2+} on global protein structure with micelle formation have been suggested by several physical chemical studies. In particular, studies using SAXS (Kumosinski *et al*., 1988) predicted a swelling of the outer shell of casein submicelles as they are incorporated into reformed micelles. This swelling represented a 30% increase in hydration (with a concomitant decrease in the electron density of proteins). Holt and Sawyer (1993) suggested that a recurrent motif in ruminant caseins is helix-loop-helix in which the loop region is typically phosphorylated. Studies of the molecular dynamics of the α_{S1}-casein phosphopeptide also suggested that the swelling of these loop structures accommodated the increased hydration that accompanied Ca^{2+} binding (Kumosinski and Farrell, 1994). Thus, presumably the Ca^{2+} binding and the incorporation of Ca caseinates into micelles could deform α-helical elements and extend loop elements because they are spatially adjacent. Hence, the changes in the $1{,}655 \pm 5\ cm^{-1}$ region of the FTIR spectra upon Ca^{2+} addition could be due to loop-helix alterations with movement of the resonances to higher wave numbers and with the higher bond energies needed to buttress the swelling and extension of the polypeptide chains.

All of the above data point to the loop-helix-loop motif as being flexible and subject to conformational change on ligand binding and conversion to aggregate structures. This is in contrast to the apparent rigidity of the hydrophobic peptides studied in Sections 5.3, 5.4, and 5.5. How then can we reconcile these divergent views of different portions of the same molecule and in the overall framework structure of sodium caseinate aggregates (submicelles) and micelles?

5.8 The Tensegrity Hypothesis and Resolution

The architectural world has often borrowed structural forms from biological shapes. Now it appears that an architectural contrivance may help us to understand biological forms. Tensegrity structures were originally constructed by Snelson and popularized by Buckminster Fuller as the geodesic dome (Ingber, 1998). In architectural parlance, the forces of compression and tension balance, so that rigid struts bear compression while more flexible elements stretch. Tensegrity structures offer the maximum space (openness) for a minimal amount of building material. A simple toy shown in Fig. 5.12 best illustrates the

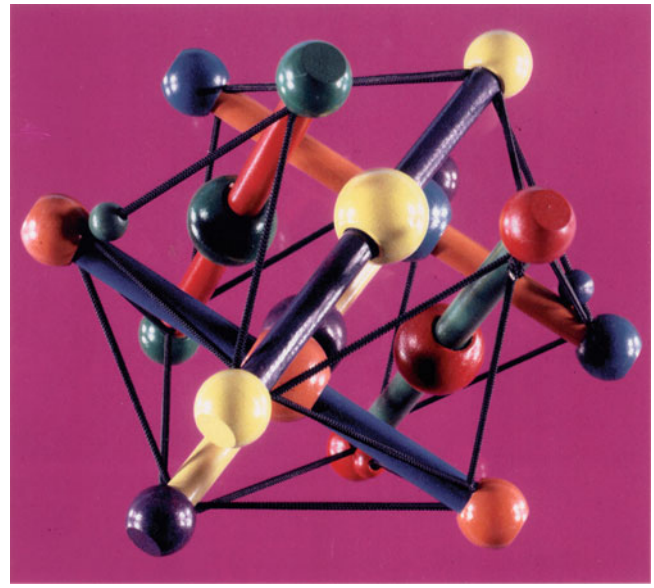

Fig. 5.12 Typical tensegrity structure illustrating the principles of rigidity and flexibility, published by permission of Manhattan Toy, Minneapolis, MN, 55401

concepts of tensegrity structures which are of interest to us. Donald Ingber has pioneered the application of tensegrity concepts to biological structures ranging from the cytoskeleton of cells to microtubules, to viral envelopes, and to pollen grains (Ingber, 1998). We propose, here, that a type of tensegrity structure may account for the overall properties of sodium caseinate aggregates (submicelles) and casein micelles.

In the putative submicelle framework shown in Fig. 5.9, the protein–protein interactions which lead to this structure occur primarily *via* sheet-turn-sheet interactions. In computer experiments, a good deal of structural integrity was gained through these interactions (Kumosinski *et al*., 1994c). As we have seen, these structures, which follow the sheet-turn-sheet motif, occur in α_{S1}-casein monomers and dimers and are quite thermally stable in solution (Alaimo *et al*., 1999b) and under molecular dynamic simulations (Malin *et al*., 2005). For contrast, consider the high degree of flexibility which occurs for the helix-loop-helix motif relative to the sheet-turn-sheet motif, once the α_{S1}-dimer has been formed. κ-Casein also contains this same rigid motif (Fig. 5.7) between residues 14 and 64; this sheet-turn-sheet motif also appears quite heat stable in κ-casein as well (Table 5.5) and in isolation it gives rise to amyloid structures. Note also that Graham *et al*. (1984) and Farrell *et al*. (2001) demonstrated a good deal of heat stability for sheet and turns in β-casein by similar CD studies. In addition, the tailspike peptide is another example of sheet-turn-sheet interactions translated into β-casein. In the tensegrity structural analogy we propose that these heat-stable sheet-turn-sheet motifs of α_{S1}-, β-, and κ-caseins interact as shown in Sects. 5.3, 5.4, and 5.5 and represent the solid struts or pre-compressed modules (Fig. 5.12). These structures then provide the framework for casein–casein interactions. For β-casein, these interactions are more hydrophobic and in the previous 3D models (Kumosinski *et al*., 1994b, c), the β-casein was fitted into the framework of α_{S1}-/κ-casein. This also accommodates the ability of β-casein to dissociate from aggregates and micelles at 5°C (Downey and Murphy, 1970).

On the other hand, the helix-loop-helix portions of the α_{S1}- and β-caseins represent more flexible structures and are analogous to the cables of tensegrity structures. Here, changes in structure with the degree of phosphorylation, or

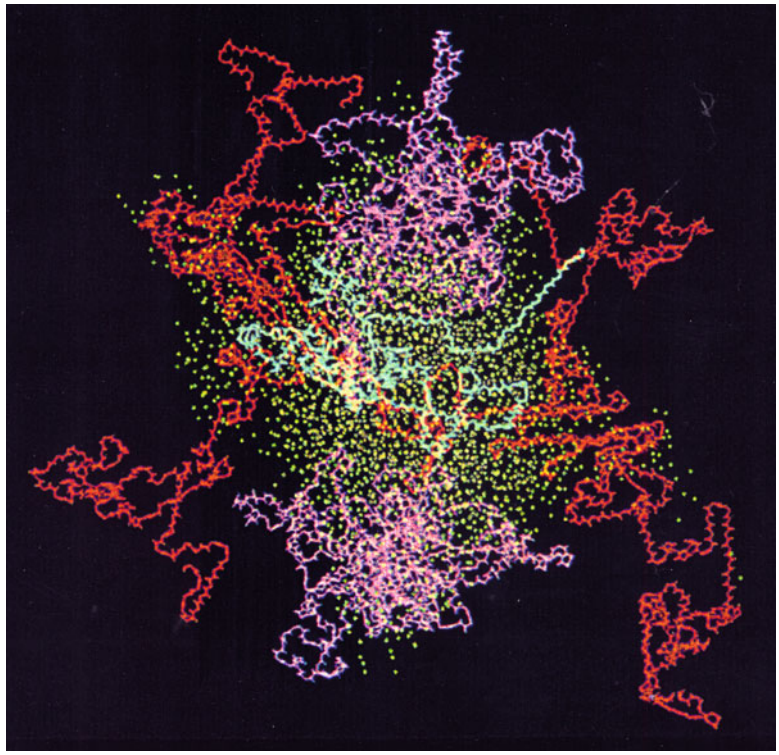

Fig. 5.13 Backbone asymmetric structure of a casein submicelle with water molecules from droplet algorithm, i.e., 2,823 water molecules: κ-casein in *blue*, α_{S1}-casein in *red*, β-casein in *magenta*, oxygen from droplet waters in cyan (Kumosinski *et al*., 1994c)

with calcium or proton binding, may yield conformational changes (Huq *et al*., 1995; Curley *et al*., 1998) or increased conformational flexibility (Kumosinski and Farrell, 1994). Halfmann and Lindquist (2010) have pioneered the concept that binding to and modification of flexible proteins (prions) may lead to significant changes in biological function. The tensegrity analogy easily accounts for the swelling of submicellar structures through flexible elements as they are incorporated into reformed micelles (Kumosinski *et al*., 1988). Once again, the principle of maximum space with minimum building material is a tensegrity concept.

For casein, then, persistent secondary structures, such as the sheet-turn-sheet motifs, define their self-association reactions, but because the tension and flexibility compromise, no hydrophobic compression occurs and the proteins remain open. In contrast the highly flexible regions such as the loop-helix-loop regions define their ability to bind and effectively transport calcium as casein micelles.

Finally, a remembrance of an old casein structure idiom: all good models hold water (M.P. Thompson). The openness of the tensegrity structures and the 3D models can readily accommodate extremely high water content in the interior space. The water content of micelles and submicelles varies from 1 to 8 g H_2O g^{-1} protein (Kumosinski *et al*., 1988). In a series of studies on casein–water interactions, Mora-Gutierrez *et al*. (1997) used ^{17}O NMR to probe and enumerate sources of bound, trapped, and preferentially absorbed water molecules. These cavities and voids have been correlated with the 3D model of Kumosinski *et al*. (1994c) as shown, partially hydrated, in Fig. 5.13. Thus, the tensegrity

hypothesis can account for the physical and chemical properties of caseins, while explaining how the molecules can be both rigid and flexible at the same time and exhibit highly hydrated backbones.

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Casein Micelle Structure, Functions, and Interactions

6

D.J. McMahon and B.S. Oommen

6.1 Introduction

Casein micelles are particles of colloidal size that can be described as supramolecules or a system consisting of multiple molecular entities held together and organized by means of non-covalent intermolecular binding interactions. The existence of such a colloidal particle consisting of a mixture of calcium phosphate stabilized by calcium-insoluble proteins has long been recognized (Linderstrøm Lang 1929). These supramolecules serve as the prime nutritional source of calcium, phosphate, and amino acids to meet the growth and energy requirements of mammalian neonates and have the biological function of transporting calcium phosphate without calcification through the mammary milk system (Horne 2002a, b; de Kruif and Holt 2003). Numerous models have been proposed to explain the supramolecular structure of casein micelles and these have been reviewed repeatedly (e.g., Bloomfield and Morr 1973; Farrell 1973; Garnier 1973; Swaisgood and Brunner 1973; Thompson and Farrell 1973; Slattery 1976; Schmidt 1980; McMahon and Brown 1984; Rollema 1992; Holt 1992; Holt and Horne 1996; Walstra 1999; de Kruif and Holt 2003). Recent insightful evaluations of the various models of casein micelle structure and the process by which this supramolecule is assembled can be found in Farrell et al. (2006a), Horne (2006) and Dalgleish (2011). In this chapter, we aim to discuss the supramolecular structure of casein micelles on the basis of investigations using electron microscopy (McMahon and McManus 1998; Oommen 2004; McMahon and Oommen 2008), interpreted in terms of their known physical and chemical attributes. The term "casein micelle" has been used in a generic sense for the calcium-phosphate-protein colloidal particles in milk for many years, but it is now very apparent that the supramolecular structure of these colloidal particles varies depending on pH, cooling, heating, and addition of other ingredients to milk. We have reserved use of casein micelle as a descriptor for these supramolecules as they are synthesized and secreted from the mammary gland, i.e., native casein micelles in non-cooled raw milk. The model structure described in McMahon and Oommen (2008) was based on observations of casein micelles from bovine milk although it appears that casein micelles from milk of other species are similar.

In bovine milk, the caseins consist of four major proteins, α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein that are secreted in their numerous genetic and posttranslational variations (Chaps. 4 and 15). The calcium-sensitive caseins (α_{s1}-, α_{s2}-,

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and β-caseins) are members of a single-gene family as seen from the homologous gene sequences of different species while the κ-casein gene is homologous to γ-fibrinogen (Swaisgood 1992). Caseins undergo posttranslational phosphorylation to varying degrees at seryl residues (see Chap. 4).

The secondary structure of the caseins has often been referred to as random coil, although this is misleading and caseins as a group of proteins can be expected to be as highly adapted to their biological function as any other structural protein (de Kruif and Holt 2003). A better description is to consider the caseins as being intrinsically unstructured proteins (Farrell et al., 2006b; Chap. 5) similar to other secretory Ca-binding proteins (Smith et al., 2004). Their physiological function in the mammary gland results from their different partially folded conformations and from structural transitions between them. Other terms used to describe the considerable conformational flexibility of the caseins include molten globule structure (Malin et al., 2005) and rheomorphic structure (Holt and Sawyer 1993).

Farrell et al. (2006b) predicted that α_{s1}- and α_{s2}-caseins are natively unfolded proteins with extended coil-like (or pre-molten globule-like) conformations, whereas β- and κ-caseins would possess molten globule-like properties. Proteins in a molten globule state have a compact structure with a high degree of hydration and side chain flexibility; they possess native secondary structures with little tertiary folds. Because of their lack of a fixed three-dimensional tertiary conformation, caseins can react very rapidly to environmental changes. This ability to exist in various conformations was described by Holt and Sawyer (1993) as the caseins being rheomorphic. In the mammary cells, caseins function by sequestering small clusters of calcium phosphate, thus preventing precipitation and calcification of the mammary milk synthesis and transport system (Horne 2002a; de Kruif and Holt 2003). This conformational flexibility further allows them to interact with multiple target molecules, and in that sense, caseins can be considered as part of the scavenger class of unfolded proteins (Smith et al., 2004). Such conformational flexibility is biologically important in binding to surfaces and the formation of macroscopic networks. Maintaining such flexibility helps explain (de Kruif and Holt 2003) the slightly nutritionally suboptimal amino acid composition of the Ca-sensitive caseins since their evolutionary imperative is preventing pathological precipitation of calcium phosphate in the mammary gland. This structural flexibility of the casein molecules is then inherently passed on to the supramolecule level and can be observed in the response of casein micelles to different environments. Whether considered as being rheomorphic or having a molten globule structure, conformational flexibility of the caseins seems critical for the casein supramolecule to maintain its structure. Various structures for the casein micelle are possible based on how they are synthesized and chemical treatments to which they are subjected. Structural information presented in this chapter will primarily be focused on the supramolecular organization of native casein micelles.

6.2 Modeling the Casein Micelle

It is interesting to look at the evolution of models of casein micelles as our understanding and ability to analyze casein systems has increased. The core-coat model proposed by Waugh and Noble (1965) consisted of spherical particles of α_{s1}- and β-caseins with κ-casein as the coat. The model put forward by Payens (1966) showed a considerable portion of κ-casein located on the periphery of the casein micelle and compactly folded α_s-caseins attached to loose β-caseins as the core, with calcium ions interacting with phosphate or carboxylic acid groups of the proteins. Bloomfield and Morr (1973) postulated the existence of a size-determining supramolecule framework predominantly made of α_{s1}-casein with β- and κ-caseins attached to the framework and filling its interstices through Ca bridges. This was based on partial calcium depletion in which β- and κ-caseins dissociate leaving a framework having the same frictional resistance as the original supramolecule. Rose (1969) proposed a model in which end-to-end β-casein association initiated supramolecule formation to which α_s- and κ-casein

molecules were in turn bound to form a protein aggregate. In the presence of calcium, these were cross-linked by calcium phosphate to form the supramolecule. Garnier and Ribadeau-Dumas (1970) proposed a model based on the aggregation behavior of the different caseins with trimers of κ-casein acting as the branching nodes and α_s- and β-caseins as the branches to form a porous supramolecule.

At about the same time, and on the basis of sedimentation behavior of urea- and oxalate-treated casein micelles, Morr (1967) put forth the subunit model in which the subunit core was made of a β-α_s-casein complex surrounded by an α_s-κ-casein complex with the subunits held together by calcium and colloidal calcium phosphate linkages. Schmidt and Payens (1976) modified this model by proposing subunits with a hydrophobic core surrounded by a hydrophilic coat of carboxylic and phosphate groups. As proposed by Morr (1967), this model also included calcium, magnesium, and colloidal calcium phosphate groups as the linkage between the subunits. Slattery and Evard (1973) proposed that κ-casein was localized on particular regions of the submicellar surface, thus forming two distinct regions which are hydrophilic and hydrophobic. Aggregation of the subunits occurred by hydrophobic bonding until the whole supramolecule surface was covered with κ-casein. However, this model did not include any function of colloidal calcium phosphate in the stability of the casein supramolecule.

Schmidt (1980) and later Walstra (1990) improved on this model by postulating that those surface regions of the submicelles not covered with κ-casein consisted of polar moieties of other caseins, e.g., their phosphoserine residues. The subunits aggregated together *via* the colloidal calcium phosphate attached to α_{s1}-, α_{s2}-, and β-caseins. As in the model suggested by Slattery and Evard (1973), casein supramolecular growth terminated when the colloidal particle surface was covered with κ-casein. Walstra (1999) modified the submicellar model of casein micelles to include calcium phosphate packages to be placed not only on the surface of submicelles as the bridging between them but also inside the subunits. However, none of these models could explain the dissociation behavior of casein supramolecules on treatment with excess κ-casein or urea (Holt 1998).

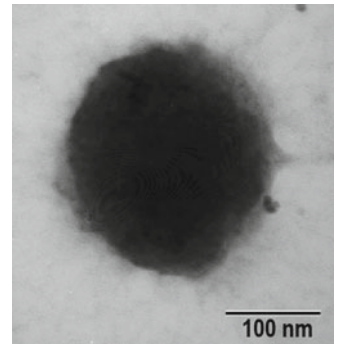

Fig. 6.1 Transmission electron micrograph of a casein micelle obtained from bovine milk that was been treated with glutaraldehyde followed by poly-L-lysine immobilization onto a parlodion-coated copper grid, then stained with uranyl oxalate, rapidly frozen and freeze-dried. Reprinted from J. Dairy Sci. 91:1709–1721 with permission from American Dairy Science Association

Support for a supramolecular structure of casein micelles consisting of subunits had come from chemical and physical measurements of casein micelles and their dissociation into smaller particles, by analysis of particles (~20-nm diameter) made from sodium caseinate, and their subsequent growth into larger supramolecules (~100- to 20-nm diameter) upon addition of calcium. Early electron micrographs generally showed a corpuscular and globular appearance of casein micelles but more recent work has shown that the supramolecular structure of the casein micelle is quite sensitive to its environment such that artifacts are easily introduced during sample preparation (McMahon and McManus 1998). When viewed using a transmission electron microscope, casein micelles that have been glutaraldehyde-fixed appear quite electron dense, compact, with an internal quaternary structure that could not be distinguished (Fig. 6.1). This is even more problematic when using an electron microscopy method that involves metal coating. The additive nature of glutaraldehyde fixation has the potential to fill interstices between proteins with polymerized glutaraldehyde. Such material would appear transparent using transmission

electron microscopy (TEM) compared to proteins (or calcium phosphate) that have been stained with heavy metals. In comparison, any metal coating used in scanning electron microscopy or replica formation would cover both proteins and cross-linked glutaraldehyde. The ion beam etching observations of Hojou et al. (1977) that strongly supported the submicelle model can then be explained as the ion bombardment etching away the accumulated glutaraldehyde and allowing a metal coating to be applied directly to protein chains.

A polyelectrolyte brush model of casein micelles in which the caseins randomly associate to form an entangled structure within which nanoclusters of calcium phosphate are held has also been proposed (Holt 1992; Holt and Horne 1996). The binding of caseins to pre-crystalline calcium phosphate nanoclusters prevents their nucleation and growth and thus blocks calcification of the mammary tissue. In this model, the binding of caseins to calcium phosphate initiates the formation of the casein supramolecule (de Kruif and Holt 2003). However, as described by Horne (2006) these two processes of protein polymerization and calcium binding of casein molecules would occur simultaneously.

Farrell et al. (2006a) proposed that the formation of small casein aggregates during casein supramolecule synthesis within Golgi vesicles occurs prior to formation of calcium phosphate nanoclusters. This was based on the sequence of events occurring during bioassembly of casein micelles in the mammary gland. In this scenario, the caseins are synthesized on ribosomes of the rough endoplasmic reticulum and then undergo folding and association (both self and mixed association are possible) that helps them escape degradation. Transportation *via* vesicles at the Golgi apparatus then occurs in which Farrell et al. (2006a) propose that the sequence of events is (1) an increase in calcium concentration in the vesicles, (2) phosphorylation of the caseins when the calcium concentration is sufficiently high (kinase K_M ~20 mM), and (3) further secretion of calcium phosphate into the vesicles. The protein aggregates were reported to be spherical complexes of about 10 nm diameter that were present in the vesicles as branched and linear chains (Farrell et al., 2006a). This, however, is only slightly larger than the presumed size of monomeric casein (i.e., Stokes radius ~4 nm) and smaller than their polymeric forms (e.g., native κ-casein polymers with Stokes radius of ~10 nm) (Swaisgood 2003). In similar studies of lactating mammary glands, Helminen and Ericsson (1968) observed that branching fibrils of protein (8–9 nm thick) initially formed in the Golgi vacuoles, then accumulated to form, what they termed, the mosaic pattern of the casein micelles, and were excreted into the lumen. Such fibril formation provides greater support for a framework rather than subunit model of the casein micelle supramolecule.

While the subunit model was widely accepted in which clustering of casein molecules into small subunits which then further clustered to form the secreted colloidal particle (McMahon and Brown 1984; Rollema 1992), such a particulate internal structure of casein micelles has not been observed (McMahon and McManus 1998; Holt et al., 2003; Dalgleish et al., 2004). Rather, observations during dissociation experiments and scattering data of simulated casein micelles provide the most support for presence of submicelles. Such experiments do not usually include microstructural observations in addition to measurement of physicochemical parameters. From small-angle X-ray scattering, Kumosinski et al. (1988) studied the supramolecular structure of casein micelles synthesized by adding calcium to sodium caseinate and deduced that the submicelles consisted of a spherical hydrophobic core with a loose hydrophilic shell with the submicelles being held together through calcium phosphate linkages with overlap of hydrophilic regions of the proteins in adjacent submicelles.

McMahon and McManus (1998) argued that previous electron microscopic images of casein micelles do not represent the native casein micelle supramolecule as the sample preparation needed in most electron microscopy introduces artifacts into the imaged structure. A more representative image of casein micelles was that they comprise strands of electron-dense regions, no more than 8–10 nm in length. Variations in scattering intensities from X-ray and neutron scattering would

then not imply an internal repeating substructure but rather a heterogeneous internal structure. Pignon et al. (2004) concluded that X-ray scattering by casein micelles could be attributed to globular supramolecules (with radius of gyration of 102 nm) and the open conformational structure of the supramolecules' constituent proteins (with radius of gyration of 5–6 nm) rather than from any globular submicelles. Dalgleish (2011) concluded that the various X-ray and small-angle neutron scattering data could be best interpreted as being a result of calcium phosphate nanoclusters rather than varying density of protein. A polycondensation model proposed by Horne (1998) envisaged cross-linking of individual caseins through hydrophobic regions of the caseins and bridging involving clusters of calcium phosphate for the assembly of the casein supramolecule. This was later described as a dual-binding model (Horne 2002a) in which growth of hydrophobically bonded proteins is inhibited by electrostatic repulsive interactions, whereby total interaction energy can be considered to be the sum of electrostatic repulsion and hydrophobic interaction. According to this model, κ-casein is linked into the casein supramolecule by hydrophobic bonding of its N-terminal region and, therefore, further growth beyond the κ-casein is not possible as it does not possess either a phosphoserine cluster for linkage *via* colloidal calcium phosphate or another hydrophobic anchor point to extend the chain.

6.3 Physical Properties

Casein micelles are highly hydrated and sponge-like colloidal particles. Of the approximately 4 g water/g protein contained within the supramolecule, only about 15% is bound to the protein, the remainder being simply occluded within the particle (de Kruif and Holt 2003; Farrell et al., 2003). Supramolecule size distribution had been reported as extending from 20 to 600 nm diameter with a median size between 100 and 200 nm (Schmidt et al., 1974; Bloomfield and Mead 1975; de Kruif 1998), depending on whether the method used to measure particle size generates a

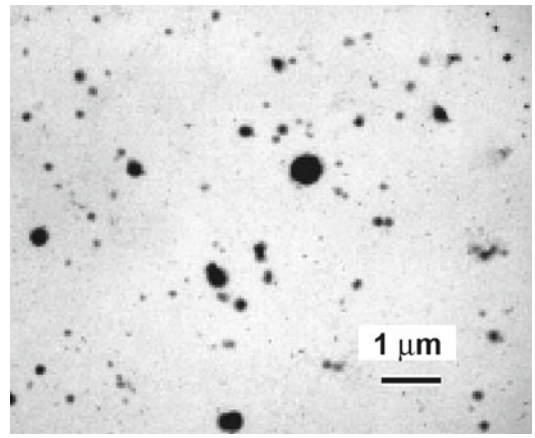

Fig. 6.2 Low magnification transmission electron micrograph of field of view of casein micelles obtained from bovine milk by poly-L-lysine immobilization onto a parlodion-coated copper grid that was then stained with uranyl oxalate, rapidly frozen, and freeze-dried. Reprinted from J. Dairy Sci. 91:1709–1721 with permission from American Dairy Science Association

number or weight average diameter (Udabage et al., 2003). Using a number distribution yields a median diameter of 1.1×10^2 nm while a weight distribution yields a median diameter of 2.2×10^2 nm. The presence of some casein micelles being up to 7×10^2 nm in diameter underlies the broad size distribution (de Kruif 1998) typically observed (Fig. 6.2) for casein micelles. Although when considered on a number basis, the particle-size distribution can be represented by a log-normal distribution (Udabage et al., 2003).

Early measurements of casein micelle size (Schmidt et al., 1973) had also reported particles less than 20 nm in diameter accounting for 80% of particles by number (but only 3% by volume). More recent measurements (de Kruif 1998; Udabage et al., 2003) infer that ~50 nm diameter is the lower end of particle-size distribution, and large numbers of very small casein micelles have not been observed in electron microscopic images of milk (McMahon et al., 2009). Such polydispersity is common in colloidal systems although biological systems tend to be more homodisperse (Bloomfield 1979) and is indicative that the physiological function of casein micelles is independent of particle size. The number of protein

molecules that constitute the casein supramolecule is about 10^4 for a colloidal particle of ~150 nm diameter (Kirchmeuer 1973). Given their range in diameter from 50 to 700 nm, the structure of casein micelles is such that it allows for synthesis in the mammary gland of supramolecules containing 10^3 to 10^6 casein molecules.

6.4 Thermodynamic Forces

A variety of forces that derive from the chemistry of the caseins (Chap. 4) come into play in preserving the structural integrity of casein supramolecules. These include hydrophobic-related interactions, ionic and electrostatic interactions, hydrogen bonds, disulfide bonds, and steric stabilization. At low temperatures, the contribution of entropy to free-energy change becomes less important, allowing proteins that are primarily linked *via* hydrophobic interactions to migrate from the supramolecule. β-Casein and κ-casein diffuse out of the micelles at low temperatures more so than α_{s1}- and α_{S2}-caseins, suggesting a greater proportion of the β- and κ-casein molecules are held into the lattice structure of the casein supramolecule solely by hydrophobic interactions. Similar involvement of hydrophobic regions of the proteins has long been recognized in casein polymerization with α_{s1}/κ-casein copolymers being formed by hydrophobic rather than electrostatic interactions (Dosako et al., 1980). A consequence of interaction of the highly phosphorylated caseins (α_{s1}- and α_{s2}-caseins, and to some extent β-casein) with calcium phosphate nanoclusters is that it would segregate these caseins from the singly phosphorylated κ-casein during synthesis and dissociation.

Many sites for ionic bonding exist within the different caseins that can play a role in casein supramolecule stabilization. In addition to the phosphoserine side chains of the calcium-sensitive caseins, there can also be electrostatic interactions between carboxylate residues and calcium (and any other divalent metal ions present in milk). These can help impart structural stability to the casein supramolecule by providing calcium cross-linking between proteins in addition to their interactions with the calcium phosphate nanoclusters. Destabilization of casein micelles near their isoelectric pH shows that ionic interactions are important for supramolecule stabilization. As milk is acidified there is a decrease in magnitude of net negative charge on individual protein molecules and the overall net charge of casein supramolecules (Heertje et al., 1985). Solubilization of calcium phosphate also causes calcium phosphate inside the casein supramolecule to be gradually depleted. Any protein molecules (such as some α_{s1}-, α_{s2}-, and β-caseins) that are bound into the supramolecule structure only by the sequestering action of their phosphoserine groups toward the calcium phosphate can dissociate especially if milk is cold and hydrophobic interactions are minimized.

For casein micelles, steric stabilization is provided by having an increased proportion of κ-casein on the periphery of the colloidal supramolecule. The highly hydrophilic glycomacropeptide portion of κ-casein (residues 106–169) provides stability against aggregation (McMahon and Brown 1984). When these peripheral protein segments on separate particles interpenetrate, or compress each other upon close approach, there is a loss of entropy due to restriction in their configurational freedom and a positive free-energy change from the increased protein segment concentration. Such steric repulsion is a short-range force and depends on magnitude of solvation forces around the protein, and for native casein micelles, it is sufficiently strong to overcome the dispersion force of attraction (Horne 1986; Walstra 1990; Holt 1992; Holt and Horne 1996).

6.5 Casein Polymerization

The polymerization (aggregation) behavior of caseins is based on the possibility of calcium-mediated interactions *via* clusters of phosphoserine groups (Dalgleish and Parker 1979), interactions *via* hydrophobic regions, interactions with water *via* their hydrophilic regions (Yoshikawa et al., 1981), as well as hydrogen bonding and the various electrostatic interactions (such as calcium bridging between negatively

charged sites and ion pairing) that are common to all proteins. Because of the varied ways in which the caseins can interact with themselves (homopolymers and aggregates), with each other (heteropolymers and aggregates), and with minerals (e.g., ionic Ca and calcium phosphate nanoclusters), there is a range of functionalities over which they can interact depending on their surrounding environment. β-Casein and κ-casein form soap-like micelles with a degree of association of 23 and 30, respectively (Payens and Vreeman 1982); in the absence of calcium (Swaisgood 2003), α_{s1}-casein forms tetramers and subsequent linear polymers (Payens 1966), while β-casein can form linear polymers of indefinite size (Payens and Markwijk 1963). Both α_{s1}- and β-casein form mixed complexes with κ-casein (Garnier et al., 1964; Payens 1968; Garnier 1973), and they can interact and polymerize to different degrees under various conditions of pH, ionic strength, and temperature.

In the absence of calcium, Thurn et al. (1987) reported that at high ionic strength, α_{s1}-casein forms polymer-like chains with its hydrophobic regions joined end-to-end. Malin et al. (2005) found predominately dimers for all three genetic variants of this protein at 37°C and at physiological ionic strength. All of the caseins can form some type of self-association structure when in solution, but their association in a mixed system containing calcium phosphate is more complex, as this increases their functionality (*f*) or number of possible simultaneous polymer interactions that can occur.

Dalgleish and Parker (1979) assigned an average $f=2$ for calcium-induced aggregation of α_{s1}-casein based on four to ten calcium ions being bound per protein molecule. Horne et al. (1988) assigned chain-terminating ($f=1$), bifunctional ($f=2$), and trifunctional ($f=3$) roles to κ-, β-, and α_s-caseins, respectively, although it may be more appropriate to assign α_{s1}-casein as $f=2$ and α_{s2}-casein as $f=3$. Within casein micelles there are opportunities for both specific phosphoserine-mediated interactions with calcium phosphate nanoclusters and hydrophobic and ionic interactions with other proteins. These interactions can also be viewed as the caseins being block copolymers consisting of blocks with high levels of hydrophobic or high levels of hydrophilic amino acid residues (Horne 2002a; Euston and Horne 2005).

As described by Horne (2002b), β-casein can act as a duo-block polymer that can interact with other proteins *via* calcium bridging because of its cluster of phosphoserine residues as well through its hydrophobic region. α_{s1}-Casein could interact as a tri-block polymer through predominantly hydrophobic regions at its C- and N-terminals, as well as a hydrophilic-rich region that contains its phosphoserine clusters. α_{s2}-Casein can be considered a hybrid of α_{s1}-casein and β-casein because, starting from its N-terminal, it can interact through two sets of alternating hydrophobic-rich and hydrophilic-rich regions and thus has the possibility of acting as a tetra-block polymer. κ-Casein would act primarily as a monoblock unit because it lacks a cluster of phosphoserine residues and the glycosylation of its hydrophilic C-terminal prevents any strong electrostatic interactions with other proteins except via its N-terminal hydrophobic region. It is known to be present in milk as disulfide-linked oligomers with other κ-casein molecules (Rasmussen et al., 1999) and either alone or as an aggregate it can be considered as a polymerization terminator. Dalgleish (2011) described the role of κ-casein in casein micelle synthesis as being similar to a surfactant-limited polymerization reaction in that since κ-casein is not involved in calcium phosphate nanocluster stabilization, it is available to limit the overall process and accumulates on the supramolecule periphery.

Colloidal size aggregates similar in size to casein micelles can be synthesized using α_{s1}-, α_{s2}-, β-, and κ-caseins, along with calcium, phosphate, and citrate as components (Schmidt et al., 1974, 1977; Slattery 1979). Though they may seem to be similar to native casein micelles (Knoop et al., 1979), their structure may vary depending on the degree of casein hydration, pH, salt balance, and the forces contributing to its integrity. When a sodium caseinate solution was examined according to McMahon and Oommen (2008), the proteins adsorbed onto the parlodion-coated grids were observed at low magnification

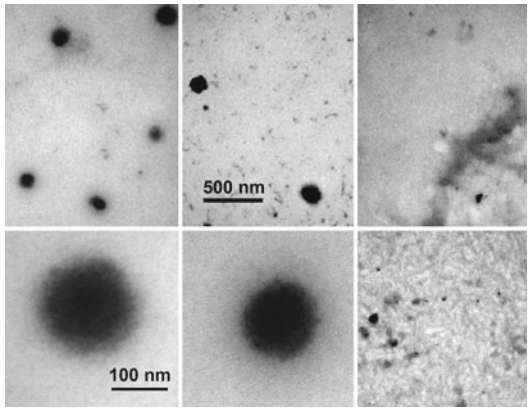

Fig. 6.3 Low (*top*) and high (*bottom*) magnification of protein aggregates obtained after hydrating skim milk powder (*left*), calcium caseinate powder (*middle*), and sodium caseinate powder (*right*) using a shear rate ~735 s^{-1} for 10 min followed by 60 min at 40°C, then processed as described in Fig. 6.2. Reprinted from Oommen (2004)

to be present as a gel-like structure with the proteins forming a mesh across the grid surface and no evidence of spherical colloidal structure (Fig. 6.3). In the absence of calcium, the proteins remained as strands or small agglomerates of proteins. Interestingly, these are reminiscent of the protein chains observed in Golgi vesicles by Farrell et al. (2006a) and Helminen and Ericsson (1968). This is as expected given the polymerization characteristics of the caseins and the translucent (non-milky) appearance of sodium casein solutions. Farrer and Lips (1999) predicted that self-assembly of caseins in sodium caseinate solutions would produce linear polymeric rods and weakly branched chains. For the same solids concentration, calcium caseinate has a lower viscosity than sodium caseinate solutions and appears opaque and white, as does milk.

Calcium caseinate solutions contain both large colloidal particles (approximately 300 nm diameter) and smaller particles of 10 to 20 nm diameter as observed using TEM (Fig. 6.3). They have a spherical appearance similar to native casein micelles in milk but when stained with heavy metals (e.g., OsO_4) they appear much more electron dense and blacker. This increased electron density could result from a combination of higher concentration of protein in the particles or a high level of calcium binding to the caseins. As there is no phosphate present to form calcium phosphate nanoclusters, the calcium would be expected to be distributed throughout the casein supramolecular structure and attached to all of the phosphoserine side chains of the calcium-sensitive proteins. These phosphoserine sites of calcium bridging between proteins and other negatively charged sites on the proteins not involved in bridging would then be readily available for exchange during heavy metal staining.

Given the open conformation and structure of the caseins, their flexibility and rheomorphic response to their chemical environment, it can be expected that the internal structure of casein supramolecules is dependent on the sequence of their synthesis. Both sodium caseinate and calcium caseinate have acid-precipitated casein as a starting material, either as a wet slurry or a dried acid casein powder. As discussed in more depth below, acidification of milk solubilizes calcium phosphate nanoclusters present in native casein micelles and reduces their ζ-potential such that there is a release followed by reaggregation of proteins into the colloidal particle that then undergoes coagulation through hydrophobic interactions to form the acid casein gel (McMahon et al., 2009). The supramolecular structure of such spherical particles formed during acid coagulation of milk is not expected to be the same as native casein micelles.

In manufacture of calcium and sodium caseinate, neutralization of the acid gel restores the net negative charge on the colloidal particle surface, causing the gel to disintegrate as the colloidal particles repel each other. In the absence of calcium, the proteins continue to disassemble until a stable state is reached in which short chains are all that remain (see Fig. 6.3). Calcium caseinate can be formed directly by neutralizing the acid curd with calcium hydroxide or by first neutralizing the acid curd with ammonia (or sodium hydroxide) and then adding calcium (Bylund et al., 2003). This would be similar to adding calcium to a sodium caseinate solution. When calcium chloride is added to EDTA-calcium-depleted casein micelles, the calcium binding to phosphoserine in such synthetic casein supramolecules is

different to that which occurs in native casein micelles (Gebhardt et al., 2011).

Neutralization with calcium hydroxide occurs more slowly than with sodium hydroxide (Bylund et al., 2003). Simultaneously to increasing the net negative charge, calcium bridging would occur between individual proteins within the colloidal particles, keeping them intact and preventing further disintegration of the supramolecular structure. Simulated casein micelles formed in this manner would then be expected to be a mass of entangled protein chains (similar to that described by Holt and Horne 1996) that are cross-linked through numerous calcium bridges predominantly occurring *via* phosphoserine residues of α_{s1}-, α_{s2}-, and β-caseins. However, if the acid casein is first neutralized with ammonia or sodium hydroxide and then calcium added, it would be the casein polymer chains and small particles that become the building blocks for making the simulated casein micelles. These could then be expected to have subunit attributes as shown by Pessen et al. (1989).

6.6 Interactions with Calcium Phosphate

Approximately 32 mM of calcium is present in milk in different forms that are bound (22 mM) or not bound (10 mM) to the casein supramolecule (Bloomfield and Mead 1975). Of the unbound calcium, only 3 mM are in free ionic form (Bloomfield and Morr 1973). As discussed above, calcium contained within casein supramolecules may be bound directly to phosphate ester and carboxyl groups of caseins or as part of the calcium phosphate nanoclusters that in turn are bound to phosphate esters of casein molecules. Calcium phosphate nanoclusters are considered to be an acidic calcium phosphate salt that also contains magnesium and citrate (de Kruif and Holt 2003) and probably zinc (Meyer and Angino 1977), with at least three phosphoserine phosphates also participating in the nanocluster structure (Aoki et al., 1992; de Kruif and Holt 2003).

There is a slow exchange of ions between the serum and protein-bound calcium and phosphate. In artificial casein micelles, about 50% of calcium is exchanged within 1 min and an additional 20% in 24 h (Pierre and Brule 1981; Pierre et al., 1983). The remainder is difficult to exchange (Zhang et al., 1996), and the nanoclusters are virtually bound into the casein supramolecule at the normal pH of milk (de Kruif and Holt 2003). Holt et al. (1998) proposed a core-shell model of calcium phosphate nanoclusters based on stabilization by bovine β-casein (f1–25) peptides. The nanoclusters comprise a spherical core of radius ~2.4 nm, consisting of ~355 $CaHPO_4.2H_2O$ units and surrounded by ~49 peptide chains forming a tightly packed shell with an outer radius of ~4.0 nm. Given the greater bulk of intact caseins (~4.5-nm radius) and their multiple phosphate clusters, it could be considered that calcium phosphate nanoclusters could be stabilized by binding three to five molecules of α_{s1}-, α_{s2}-, or β-casein. Calcium phosphate nanoclusters have thus been considered as having polymerization $f>4$ (Holt et al., 1996).

For α_{s1}-casein, its two phosphate centers (at amino acids 41–51 and 61–70) could act as potential regions of association with different nanoclusters (de Kruif and Holt 2003) although, as discussed by Horne (2006), these two phosphate centers on α_{s1}-casein are most likely to attach to adjacent facets of the same nanocluster. Rapid binding of caseins to calcium phosphate nanoclusters was proposed by Holt et al. (2003) as being the structure-forming points for mammary gland synthesis of the casein micelle. This would produce a calcium phosphate-protein aggregate in the size range of 7–13 nm which fits the disintegration observations by Hojou et al. (1977) and also particles observed in mammary gland Golgi vesicles during casein micelle synthesis. Although, Farrell et al. (2006a) argued that initial calcium phosphate concentrations in the Golgi vesicles are too low to allow the formation of nanoclusters prior to casein aggregation.

With phosphoserine clusters of attached α_{s1}-, α_{s2}-, or β-caseins oriented toward and participating in the calcium phosphate nanocluster, their hydrophobic domains would be oriented so they can interact with other casein molecules. Lateral hydrophobic binding to other caseins that are

attached to the same calcium phosphate nanocluster would be analogous to protein orientation and monolayer in situ polymerization that occurs when proteins are absorbed onto an oil–water interface (Dickinson and Matsumura 1991). If the hydrophobic regions are oriented in a plane away from the calcium phosphate nanocluster, hydrophobic interactions and binding could occur with other casein entities (including monomeric casein, casein homo/heteropolymers and aggregates, and caseins that are part of other calcium phosphate-phosphocasein complexes) such as described by the dual-binding approach to casein supramolecule synthesis of Horne (2002b). The interaction of caseins with the calcium phosphate nanoclusters immobilizes their flexible domains and induces rigidity in the supramolecule (Rollema and Brinkhuis 1989). If a calcium phosphate nanocluster is dissolved, the surrounding protein organization would remain intact unless the hydrophobic and electrostatic interactions between them were also disrupted.

6.7 Microstructural Imaging

The non-crystallizing nature of the individual caseins, and their aggregates, limits the use of techniques such as X-ray crystallography and multidimensional proton NMR to study their structure. Electron microscopy has thus been an important tool in deciphering the supramolecular arrangement of the caseins. The challenge has been how to prepare and view casein micelles so that the resultant electron micrographs exhibit minimal variation of the casein micelle supramolecule from its native form (McMahon and McManus 1998).

Surface images can be obtained using scanning electron microscopy without metal coating (Dalgleish et al., 2004) and cross sections of the internal structure can be seen using TEM of freeze-fractured cryo-protected casein micelle suspensions (Heertje et al., 1985; Karlsson et al., 2007). Total (surface and internal) images can be obtained by TEM of freeze-dried surface-immobilized casein micelles without resin embedding and sectioning (McMahon and McManus 1998; McMahon and Oommen 2008), by cryo-TEM of thin vitrified films of casein micelles suspension (Marchin et al., 2007), and by using cryo-transmission electron tomography (Trejo et al., 2011). When preparing casein micelles for examination by electron microscopy, it is important to realize that the integrity of these supramolecules depends on a combination of factors including strong electrostatic linkages of caseins to calcium phosphate as well as protein–protein interactions such as H-bonding, salt bridging (*via* calcium ions), ion pairing, and hydrophobic interactions (McMahon and Brown 1984). Furthermore, the casein molecule conformational flexibility that facilitates their rapid and accurate response to environmental change—the basis for their functioning so well to sequester and transport calcium phosphate in the mammary gland (Horne 2002a)—can easily lead to structural rearrangements during the chemical treatments used during sample preparation for electron microscopy.

Such structural changes are known to occur during the fixation, alcohol dehydration, and critical point drying steps of sample preparation for scanning electron microscopy. This makes interpretation of extracellular structures of biological specimens viewed at very high magnification rather limited because of uncertainties regarding artifact formation resulting from sample preparation (e.g., structural changes), surrounding materials (e.g., coatings, surface tension, ice), or image capture (e.g., contrast settings). In McMahon and Oommen (2008), a copper grid covered in nitrocellulose with a poly-L-lysine coating was used to attach a monolayer of immobilized casein micelles stained with uranyl oxalate, then immersed in liquid N_2-cooled Freon to freeze the sample without causing ice crystal formation, following by sublimation to remove the water molecules, and then viewed by TEM using a goniometer stage.

It is typical when using TEM to observe a three-dimensional spherical object such as a casein micelle that the central region of the image is darker than the periphery (Fig. 6.4). This is not indicative of a change in electron density but represents more scattering opportunities being present when the thickness of the sample traversed by

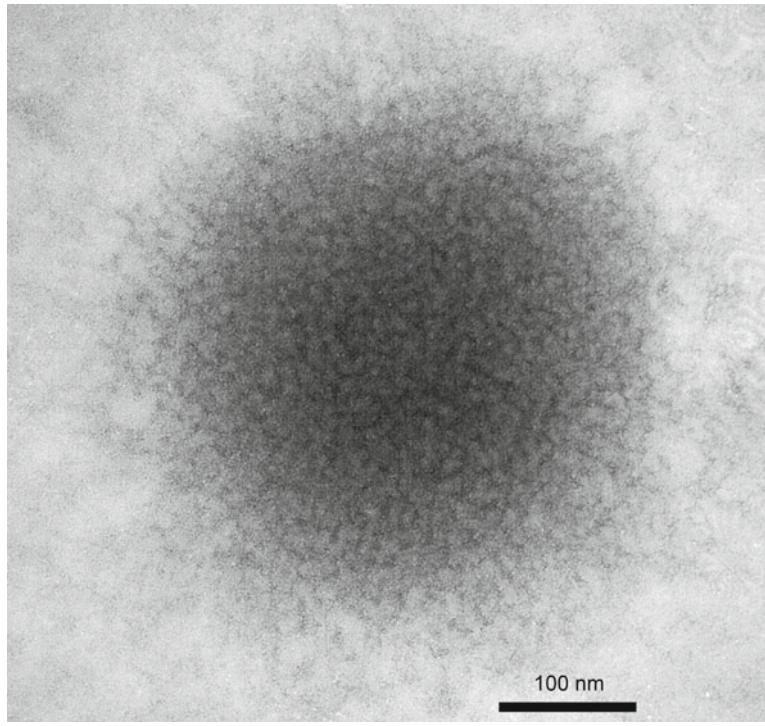

Fig. 6.4 Casein micelle from bovine milk imaged as described in Fig. 6.2. Reprinted from J. Dairy Sci. 91:1709–1721 with permission from American Dairy Science Association

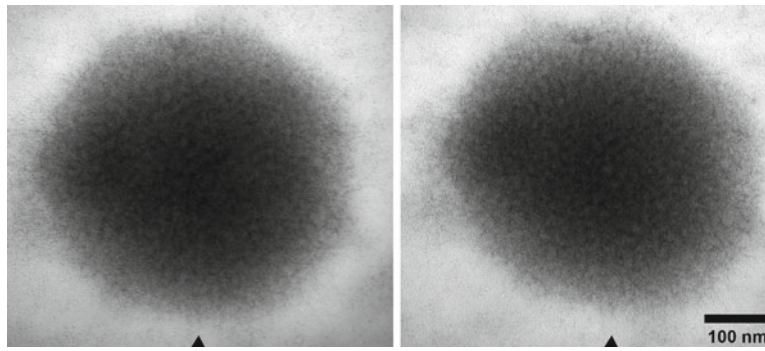

Fig. 6.5 Stereo image of a casein micelle imaged as described in Fig. 6.2 and photographed 8° apart. Reprinted from J. Dairy Sci. 91:1709–1721 with permission from American Dairy Science Association

the electron beam is greater. When viewed using stereo pairs of images (Fig. 6.5), this dimensional compression artifact can be eliminated and the supramolecular structure is observed to be uniform throughout the casein micelle. Another advantage of using stereo pairs is that because multiple images of the same casein micelle are obtained at different angles, then only objects recorded at both angles will converge giving greater confidence that what is being observed in the micrograph represents a real electron-dense entity and not an artifact related to imaging at a very high magnification. With recent advances in instrumentation and software, it is now possible to obtain internal structural images of casein supramolecules at various angles using a 140° stage rotation and then to reconstruct a three-dimensional image (Trejo et al., 2011).

It must still be recognized that there will be parts of the supramolecule that are more electron dense than others, such as the calcium phosphate nanoclusters, and where there is binding of heavy metal ions used to increase contrast. On the proteins, this occurs where they carry negative charges (carboxylate and serine phosphate sites) rather than in hydrophobic regions and where it can exchange for calcium in the nanoclusters. The contrast settings used during image capture and during image manipulation will also influence differentiation of image pixels relating to electron-dense regions and the background (McMahon and McManus 1998). It must therefore be remembered that only a selective portion of the casein molecules are imaged whether this is achieved by subjective visual adjustment or by the use of computer software. At low contrast (such as in Fig. 6.4), the extension of protein strands outward on the casein micelle periphery is retained. When viewed using stereo pairs (Fig. 6.5), the predominant appearance of casein micelles was that of electron-dense locations present as interlocked chains. Open spaces between the electron-dense locations would represent regions devoid of matter (i.e., occupied by aqueous serum in the native casein micelle) as well as portions of the proteins that have a very low electron density and cannot be distinguished from the background.

6.8 Interlocking Lattice Supramolecule

Taking into account a slight underestimation of the volume being occupied by the proteins, a model structure of the casein supramolecule was developed. When viewing an entire casein micelle (see Figs. 6.4 or 6.5), the large number of individual components being visualized (10^3 to 10^6 depending on casein micelle size) makes it difficult to view the central region of the supramolecule. There are too many overlapping planes of electron-dense locations visually to isolate individual planes. Even so, it was evident that there were no major differences among structural arrangements between the central portions of casein micelles and their peripheral regions. In McMahon and Oommen (2008) a region on the supramolecule periphery (Fig. 6.6) was selected for close examination of electron-dense entities and a digitally magnified image of this region was then visually examined stereoscopically.

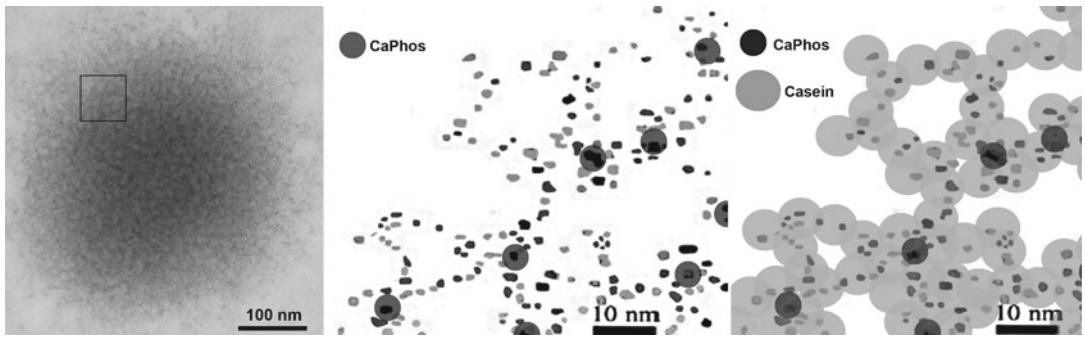

Fig. 6.6 A peripheral section of a casein micelle (*left*) imaged as described in Fig. 6.2 digitally magnified as a stereo pair and a single plane of electron-dense spots visually isolated (*middle*) with intersecting locations designated as calcium phosphate of 4.8 nm diameter, while the remainder of the electron-dense spots were considered to be casein molecules and represented as *light grey* circles of 8-nm diameter (*right*). Reprinted from J. Dairy Sci. 91:1709–1721 with permission from American Dairy Science Association

When observing a single plane of electron-dense locations, they consisted of short linear and branched polymer chains that were interlocked together on a regular basis (Fig. 6.6). At these interlocking sites there was a grouping of electron-dense locations with many near neighbors and the polymer chains appeared to radiate outward until they encountered another interlocking site. Once the skeletal structure of the supramolecule had been determined it was then necessary to make assignments to various locations based on known size and functionalities of components that make up the casein micelle. The most likely candidate for the entities forming the interlocking sites was calcium phosphate nanoclusters because of their ability to bind multiple phosphoproteins (i.e., α_{s1}-, α_{s2}-, and β-casein) and these were designated as spheres of 4.8-nm diameter as proposed by Holt and Sawyer (1993). Since it is not possible to differentiate between proteins solely on electron microscopic images, the remainder of the electron-dense locations were simply assigned as being protein with an average diameter of 8 nm. This gave the appearance of the electron-dense material forming into a lattice-type structure consisting of interlocked orbs with chains of material that encompassed areas devoid of any electron-dense material (Fig. 6.7). The presence of such channels and pools of serum throughout the interior of casein micelles has been shown recently by Trejo et al. (2011).

With this lattice supramolecule structure (Fig. 6.7), it is very apparent that casein micelles have a very open, porous structure expected for a colloidal particle with a high voluminosity in which proteins account for only 10–20% of the supramolecule volume. It is also apparent that the amount of water occluded by casein micelles is very dependent on where the surface of the colloidal supramolecule is placed and the extent of draining that occurs in these peripheral regions. As described above, the polymerization behavior of the various caseins depends on the possibility of calcium-mediated interactions *via* clusters of phosphoserine groups, the gain in entropy obtained by grouping of hydrophobic regions so as to remove them from the aqueous environment, interactions of hydrophilic regions with water, hydrogen bonding, and the various electrostatic interactions (such as calcium bridging between negatively charged sites and ion pairing) that are common to all proteins. The observed redundancy in functionality of caseins in bovine milk suggests that the range of functionalities is more important than which casein actually performs a particular function. Also, a molecule such as α_{s2}-casein with its four potential interaction regions may not always associate with other components to its maximum functionality because of steric hindrance between binding partners. Rapid binding of many caseins to calcium phosphate nanoclusters would then act as structure-forming points during casein micelle synthesis as proposed by Holt et al. (2003).

Further casein molecules can bind to structure-forming aggregates either as monomers, oligomers, or even as aggregates bound to a different calcium phosphate nanocluster. Interactions can occur *via* hydrophobic regions or calcium bridging through their carboxylate or phosphoserine side chains that are not part of the phosphoserine

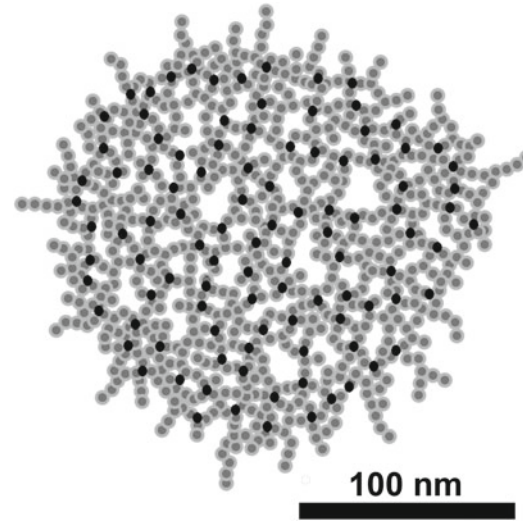

Fig. 6.7 Schematic cross-sectional diagram of the interlocking lattice model of the casein micelle with casein-calcium phosphate aggregates throughout the entire supramolecule, branched and linear chains of protein extending between them, and numerous serum pockets and channels. Calcium phosphate nanoclusters are shown with a diameter of 4.8 nm and about 18 nm apart, and caseins shown with hydrodynamic diameter of 8 nm. Reprinted from J. Dairy Sci. 91:1709–1721 with permission from American Dairy Science Association

clusters bound to the calcium phosphate nanoclusters (Swaisgood 2003). In the volume assigned to each protein molecule, multiple electron-dense locations were often observed, so this assignment is based on what we considered a representative arrangement. It should also be realized that the conformational shape of the proteins is not spherical and would to some extent depend upon interactions with their neighbors and can be in the form of compact clusters. Various structural arrangements, such as long linear chains, double-stranded chains and short branches on chains, were observed throughout casein micelles. This was expected because κ-casein, which acts as a chain terminator, is present throughout the entire casein micelle and not just on its surface.

The casein micelle supramolecule can thus be considered as an interlocked lattice (Fig. 6.7) in which the casein molecules both surround the calcium phosphate nanoclusters and extend as short chains between the interlocking points and outward at the particle periphery. The supramolecular structure is irregular and allows for a large diversity of linkages between the proteins including chain extenders (β-casein or α_{s1}-casein), chain branch points (α_{s1}-casein or α_{s2}-casein), chain terminators (κ-casein), and interlocking points (calcium phosphate nanoclusters). On the periphery of the casein micelle, there can be some chains of proteins extending outward, placing κ-casein (either individually or as disulfide-linked polymers) well out from the bulk of the casein micelle. The number of these protuberances is less than postulated in early depictions of the casein micelle surface as a hairy layer (or polyelectrolyte brush) on a hard sphere (Holt and Horne 1996) but extend further into the surrounding serum. There is no distinct hairy layer as later recognized (de Kruif and Holt 2003) and some protuberances were observed (McMahon and Oommen 2008) to be up to about 30 nm in length, which is similar in length to that observed by Dalgleish et al. (2004).

Overall, this supramolecular structure would produce a very stable colloidal particle comprising many thousands of protein molecules and hundreds of calcium phosphate nanoclusters. The distance between interlocking sites appeared similar to the 18 nm interval predicted by de Kruif and Holt (2003) for calcium phosphate nanoclusters. Some were further apart while others were closer together such that there could be from two to six protein molecules between the interlocking sites. According to Smith et al. (2004), calcium phosphate accounts for about 7% of dry mass of casein micelles, and casein micelles of 200 nm diameter and 10^9 Da contain approximately 800 calcium phosphate nanoclusters. This equates to a ratio of about 60 protein molecules per calcium phosphate nanocluster, which is more than what was observed in our electron micrographs if it is assumed that each interlocking point in the lattice structure is a calcium phosphate nanocluster. Some of the interlocking points may also result from branches in the protein chain, possibly by α_{s2}-casein.

6.9 Modifying the Supramolecule

Modification of the native physicochemical environment of milk, such as acidification, heating, blending with other fluid foods, and hydrolysis, is an integral part of manufacture of various dairy products. Understanding their influence on the supramolecular structure of casein micelles is vital in tailoring the characteristics and quality of dairy products.

6.9.1 Cooling of Milk

In TEM examination of thin sections of agar-embedded milk at different temperatures (McMahon et al., 2009), there appeared to be very few casein supramolecules at 40°C that were <80 nm diameter (Fig. 6.8). At lower temperatures (20 and 30°C) there appeared to be more electron-dense material that was only loosely attached to the supramolecules although no extensive dissociation of the supramolecules was observed. At 30°C, the supramolecules were spherical in shape but many of them had surface protuberances, and in some cases the surface

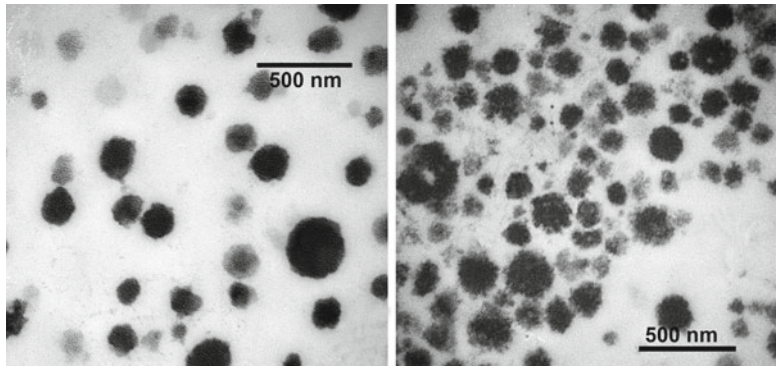

Fig. 6.8 Transmission electron micrographs of thin sections of skim milk that had been glutaraldehyde-fixed and agar-solidified at 40°C (*left*) and 10°C (*right*). Reprinted from J. Dairy Sci. 92:5854–5867 with permission from American Dairy Science Association

material seemed only loosely attached to the rest of the colloidal particle. At 20°C, the supramolecule surface had a more tendrillar appearance with electron-dense areas protruding from the particle surface.

At 10°C, the supramolecules had a ragged appearance, and there was a greater proportion of smaller particles, including some that were no longer spherical (Fig. 6.8). Also, there were some particles that had an absence of material in their core. Compared to casein micelles at 40°C, the supramolecules at 10°C were less electron dense (i.e., there was less heavy metal staining), their peripheral edges were less distinct with a relatively open structure, and there was more protein material dispersed as loose aggregates among the colloidal supramolecules. When milk was cooled to 5°C and centrifuged (27,500×*g* for 2 h), the supernatant predominantly contained small protein aggregates about 10–20 nm in size and numerous linear and branched chains of proteins (Fig. 6.9).

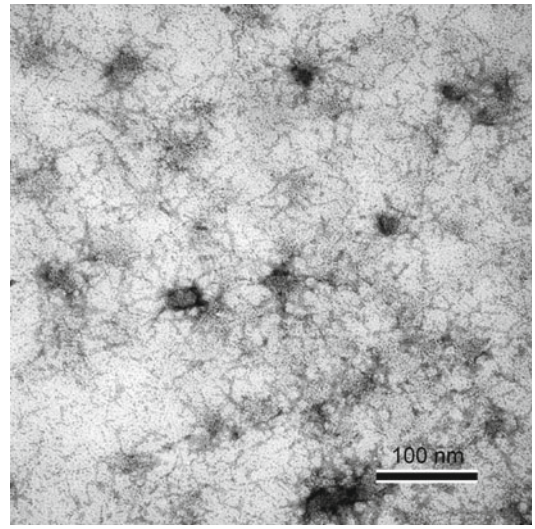

Fig. 6.9 Serum proteins obtained from the supernatant after centrifuging cold raw milk at 27,000×*g* for 2 h and imaged as described in Fig. 6.2. Reprinted from Oommen (2004)

6.9.2 Acidification of Milk

Upon acidification of milk, it is known that casein supramolecules undergo changes based on charge neutralization (Davies et al., 1977; Kalab et al., 1976) and other factors (Heertje et al., 1985; Holt and Horne 1996; Lucey 2002) that ultimately result in their aggregating into a network of chains and clusters, thus forming a gel. The physical properties of such a gel are influenced by temperature, and when cold (~4°C), the milk can be acidified without gelation occurring. Calcium removal from native casein supramolecule initially dissociates weakly bound β- and κ-caseins without any apparent change in supramolecule size (Bloomfield and Morr 1973; Lin et al., 1972). Acidification releases α_s-, β-, and κ-caseins from the supramolecule in varying proportions depend-

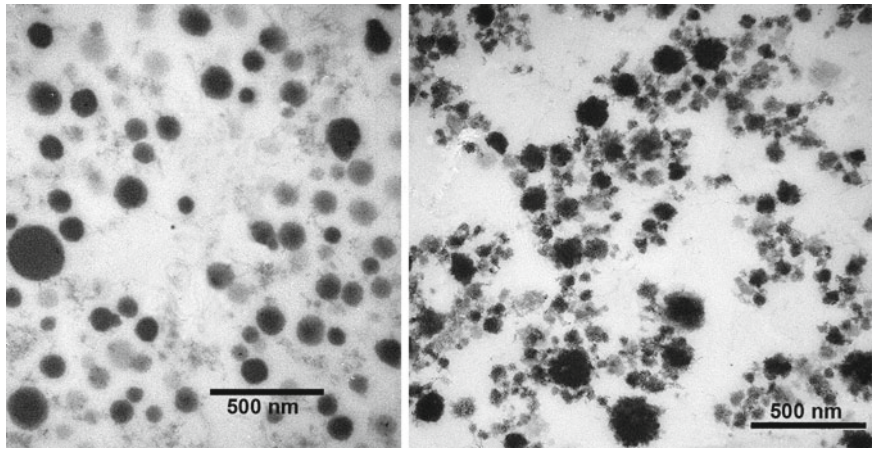

Fig. 6.10 Transmission electron micrographs of thin sections of skim milk acidified to pH 5.2 at 40°C (*left*) and 10°C (*right*) using glucono-δ-lactone then glutaraldehyde-fixed and agar-solidified. Reprinted from J. Dairy Sci. 92:5854–5867 with permission from American Dairy Science Association

ing on the experiment being conducted. β-Casein has been reported to be preferentially solubilized (Snøeren et al., 1984; van Hooydonk et al., 1986) while others (Roefs et al., 1985; Dalgleish and Law 1988; Singh et al., 1996a) reported equal dissociation of β- and κ-caseins and a lower percentage of α_s-caseins. The proteins with higher levels of phosphorylation dissociate less readily from the casein supramolecule when calcium phosphate is solubilized (Aoki et al., 1988).

Dalgleish and Law (1988) observed that at 30°C, dissociation of casein upon acidification of milk to pH 5.5 had a constant proportion of α_{s1}-, β-, and κ-casein, suggesting that these proteins may have dissociated as an intact complex. The smallness of these particles also agrees with them being enriched in κ-casein, and having less α_{s1}-casein than the original casein supramolecules. This would be expected as κ-casein is needed to stabilize the particles because neither β-casein nor α_s-caseins can exist as monomers under these conditions (van Hooydonk et al., 1986).

At low temperature there is more dissociation of proteins from the casein supramolecules during acidification than at higher temperatures. Dalgleish and Law (1988) reported 30% and 55% being dissociated at 20°C and 4°C, respectively. Singh et al. (1996b) reported only 7% and 22% dissociation at 22°C and 5°C, respectively, but they used higher centrifugal force that would sediment smaller casein supramolecules and large aggregates. McMahon et al. (2009) proposed that the first phase of acid gelation of milk involves a temperature-dependent dissociation of proteins from the casein supramolecules with less protein being released when the milk is warm (Fig. 6.10). At cold temperatures (e.g., 10°C) dissociated proteins were also present as loosely entangled aggregates. At 40°C, there appears to be sufficient hydrophobic interactions to maintain the proteins as (small) spherical colloidal particles (many with diameters <50 nm). The formation of such small spherical particles would require rearrangement and consolidation of the supramolecule interior structure with a predominance of κ-casein expected to remain on the periphery. As calcium phosphate nanoclusters that interlock the protein strands within the casein supramolecule are solubilized, the structural integrity of the casein supramolecule is weakened, promoting rearrangements inside the supramolecule as well as dissociation. It can thus be expected that the casein supramolecules would undergo a transition to compensate for the loss of interactions with calcium phosphate nanoclusters. New calcium bridging and other electrostatics protein-protein interactions would occur and hydrophobic interactions at higher temperatures.

When milk was acidified at 40°C and casein micelles captured using the method of McMahon

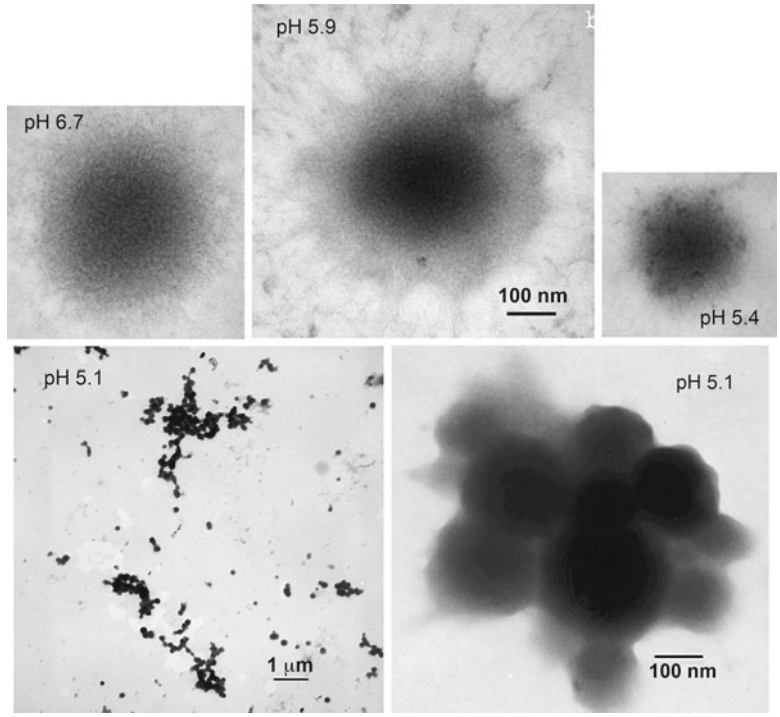

Fig. 6.11 Casein supramolecules from pasteurized skim milk (pH 6.7) and from the same milk acidified with glucono-δ-lactone and sampled at pH 5.9, 5.4, and 5.1 and prepared and imaged as described in Fig. 6.2. Reprinted from Oommen (2004)

and Oommen (2008), increased amounts of electron-dense particles were observed around their periphery; there was a loss of supramolecule integrity and shape, and large tendril appendages originated from the supramolecule and extended into the surrounding area (Fig. 6.11). By pH 5.9 the number of surface tendrils increased suggesting a progressive breakup of the supramolecule's lattice structure from its periphery into the center. There were also more smaller electron-dense nonspherical objects (ranging in size from 10 to 100 nm) in the background that correspond to the loosely entangled protein aggregates observed by McMahon et al. (2009) that had adsorbed to the grid in addition to the casein supramolecules.

As the pH was lowered, some loosely entangled nonspherical aggregates were observed (McMahon et al., 2009) to be as large as the native casein supramolecules. Casein micelle particle size has been reported (Singh et al., 1996a; Dalgleish et al., 2004) to remain relatively constant during the initial acidification of milk, but McMahon et al. (2009) observed an apparent decrease in size of the casein supramolecules with many more small casein supramolecules being present at pH 5.7. This corresponds to the pH at which Dalgleish and Law (1988) observed maximum dissociation of casein from the supramolecules at 30°C. However, the measurement of dissociated casein is actually a measure of nonsedimentable protein and this is dependent on the centrifugal force applied and whether the proteins that are released from the casein micelle remain in monomeric form or polymerize into aggregates. When large aggregates, such as the loosely entangled proteins observed by McMahon et al. (2009) are formed, they could be sedimented and incorrectly presumed to still be part of the casein supramolecules.

McMahon et al. (2009) observed considerable loosely entangled protein aggregates present in milk at 30°C that was acidified to pH 5.7 as well

as many supramolecules in the 50 to 100 nm range. At 40°C and the same pH, the loosely entangled protein aggregates were not evident but there were many small supramolecules in the 30 to 50 nm range. Oommen (2004) also observed no dissociated material observed around the casein supramolecules at pH 5.4 (Fig. 6.11). At low magnification, the supramolecules were well dispersed and in various sizes while at high magnification, small clumps of electron-dense material (10 to 20 nm diameter) were observed throughout their interior. Between pH 6.7 and pH 5.4, the dominant influences of milk acidification on protein dissociation and colloidal particle formation are a balance of temperature-dependent hydrophobic effects, calcium phosphate solubilization (which is both pH and temperature dependent), and charge neutralization. At higher temperature, the hydrophobic effect is sufficient to favor polymerization into spherical colloidal particles rather than remaining as loosely entangled aggregates.

Around pH 5.4, soluble β-casein, along with other dissociated proteins, may precipitate at their combined isoelectric point (Heertje et al., 1985) resulting in the formation of the compact aggregates of proteins observed within the casein supramolecules. As pH drops further, β-casein (theoretical pI of 5.26 at 20°C) present in such clusters would become positively charged and provide an attraction with other casein molecules in the supramolecules that are still net negatively charged. This would alter peripheral regions of the supramolecules, and as pH is lowered further all the protein polymers and aggregates would reassociate into the compact particles observed at pH 5.1 (Fig. 6.11). It may be construed that the structure of casein supramolecules at the gelation pH results from the combined influence of electrostatic interactions, hydrophobic interactions, and calcium bridging among the various caseins. This differs from native casein micelles in which the interactions between the caseins and calcium phosphate nanoclusters are a predominant contributor to its internal structure.

At 40°C, the start of milk gelation was observed at pH 5.05 (Oommen 2004) which was similar to previous reports that coagulation of pasteurized skim milk acidified at 35–50°C starts at pH 5.1–5.2 (Kim and Kinsella 1989). When milk was sampled at pH 5.1, clusters of casein supramolecules were observed (Fig. 6.11) even though the milk was still fluid. This was because these clusters can adsorb onto the grid using the method of McMahon and McManus (1998) rather than being restricted to a single plane observed using freeze etching or thin sectioning. In such cases, chains of particles or gel networks are observed only if they reside within the plane of the section (or the fracture surface). Chains of particles that cross the imaged plane may be observed as a single supramolecule or as a short chain depending on the angle at which they cross the plane and the thickness of the section (Kalab et al., 1976). The casein supramolecules at pH 5.1 (Fig. 6.11) were compact and sufficiently electron dense that internal structure could not be determined, suggesting they are more like the supramolecules in calcium caseinate and have a different structure to casein micelles as they exist in milk at pH 6.7 and 40°C.

The compact nature of the particles observed at pH 5.1 suggests that the re-association of caseins with the residual calcium-depleted casein results in a colloidal particle that not only has a ζ-potential approaching zero but whose surface lacks tendrils of protein that extend into the solvent water. Such supramolecules would lack the steric repulsion of native casein supramolecules (Tuiner and de Kruif 2002) generated by such peripheral tendrils. This combined with positively charged proteins on parts of the supramolecule periphery would allow van der Waal's attraction to overwhelm repulsive forces during colloidal particle collisions leading to aggregation and subsequent gelation.

6.9.3 Calcium Sequestration

Sequestering of calcium and dissolution of the calcium phosphate nanoclusters by agents such as EDTA can result in disintegration of the native casein supramolecules (Lin et al., 1972; Aoki et al., 1986; Holt et al., 1986). If calcium is depleted by dialyzing milk against simulated milk

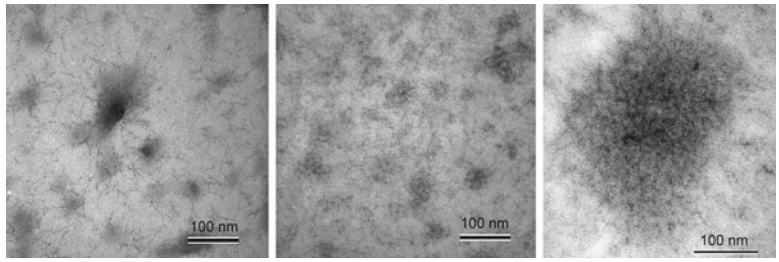

Fig. 6.12 Calcium-depleted casein supramolecules obtained after adding 43 m mol/L EDTA to bovine milk at 40°C (*left*) and 4°C (*middle* and *right*) imaged as described in Fig. 6.2. Reprinted from Oommen (2004) and from J. Dairy Sci. 91:1709–1721 with permission from American Dairy Science Association

ultrafiltrate containing EDTA, the hydrodynamic radius of those particles remains constant up to a critical level of ionic calcium reduction (Bloomfield and Morr 1973). Direct addition of EDTA into milk causes some casein micelles to dissociate completely while the rest of them still remain intact apparently depending on the extent of calcium dissociation. When calcium in milk was chelated using EDTA, the casein micelles were observed to dissociate partially and clusters of thin tentacles and strands of proteins were formed (Fig. 6.12). At 40°C there were many small filamentous aggregates that appeared to contain three to six filigreed rings of protein. In cold milk (5°C) there were clusters of particles (15–50 nm in size) and linear strands of protein that were approximately 1–2 nm in width and up to 30 nm long. These chains appeared to contain branching points or overlapped other chains. The remaining casein micelles lacked regularity in shape or size and were of low electron density. Disintegration of the casein supramolecule into smaller particles was considered as strong supporting evidence for the submicelle model. However, caseins form a dynamic system and, therefore, can rearrange themselves after disintegration. They do not exist as monomers under physiological conditions (Swaisgood 1992) so it would be expected they would remain as small aggregates.

Dialysis of casein micelles against a phosphate-free buffer also causes supramolecule dissociation and releasing predominantly β- and κ-caseins (Holt et al., 1986) with less α_{s1}- and α_{s2}-caseins. When the dialysis buffer was saturated in colloidal calcium phosphate but with ionic calcium <2 m mol/L, protein dissociation occurred while the calcium phosphate nanoclusters remained intact within the casein micelle, and Holt et al. (1986) concluded that the retention of proteins within the calcium-reduced casein supramolecule was related to their phosphoserine content, i.e., α_{s2}->α_{s1}->β->κ-casein.

Adding ionic calcium via dialysis causes transfer of soluble casein into the supramolecule without any apparent change in size of the casein micelles (Bloomfield and Morr 1973). Thus, the supramolecule structure appears sufficiently dynamic that it retains its overall structure even when some proportion of proteins are added or removed. When casein micelles are dissociated by calcium removal, the resultant particles more closely resembled sodium caseinate than small synthetic casein micelles (Rollema and Brinkhuis 1989). It appears that this is the common structure of non-colloidal caseins, such as those obtained upon cooling of milk (Fig. 6.9), in the absence of calcium and manufacture of sodium caseinate (Fig. 6.3) or after chelation of calcium by EDTA (Fig. 6.12).

6.9.4 Heating of Milk

Relatively severe milk temperature changes are involved in the manufacture of many dairy products. Heat treatment of milk above 70°C results in denaturation of β-lactoglobulin and its subsequent interaction with other denatured whey proteins and with caseins via disulfide, hydrophobic, and ionic interactions. Factors such as pH, salt

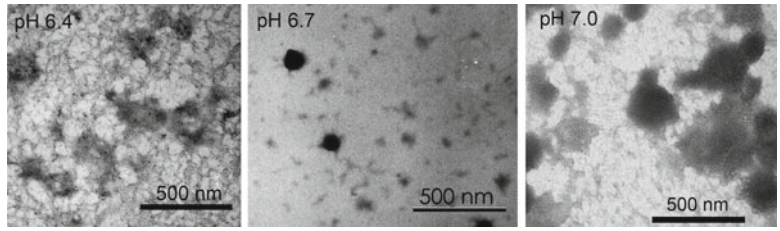

Fig. 6.13 Casein supramolecules and other proteins in milk adjusted to pH 6.4 (*left*), pH 6.7 (*middle*), and pH 7.0 (*right*) then heated to 90°C for 30 min and imaged as in Fig. 6.2. Reprinted from Oommen (2004)

system, and ionic strength and the presence of solvents and other solutes can affect such interactions with the casein supramolecules during heating. Understanding the mechanism and the influence of these treatments on structural changes of casein is vital in tailoring the characteristics and quality of dairy products. Concomitantly with heat-induced protein interactions, the pH of milk decreases as calcium and phosphate becomes less soluble and further associate with the casein supramolecules releasing H^+.

Associations of β-lactoglobulin with κ-casein through disulfide linkages is also pH dependent (Heertje et al., 1985; Corredig and Dalgleish 1996; Anema and Klostermeyer 1997). Anema and Li (2003) reported an increase in casein supramolecule size by 25–30 nm at pH 6.5 when heated at 90°C for 30 min compared to an increase of 5–10 nm at pH 6.7. Electron microscopic investigation of this heat-induced complex formation by Heertje et al. (1985) showed that at higher pH (pH ≥ 7.0), large aggregates formed that were not attached to the casein supramolecules, while at lower pH (pH ≤ 6.7) the aggregates were attached around their periphery. At pH 6.7 after heat treatment to 90°C, Oommen (2004) observed casein supramolecules as dark electron-dense particles with numerous appendages around their periphery (Fig. 6.13). These appendages were of various sizes and shapes and had the appearance of large, but less electron-dense, protein aggregates attached to proteins on the casein supramolecules similar to Heertje et al. (1985). Heating milk that had been acidified to pH 6.4 produced clumps of casein supramolecules attached to a virtual web of aggregated material. Their electron density was less than that observed at pH 6.7 suggesting that some dissociation had occurred and there were compact and electron-dense regions as well as numerous tendrils of attached protein chains and clusters of lower electron density.

When pH was increased to 7.0 and the milk then heated to 90°C for 30 min, there were large (100 to 500 nm diameter) spherical electron-dense particles (casein supramolecules) with large nonspherical aggregates of lower electron density either attached to the casein supramolecules or occupying the surrounding areas. The distinct internal filigreed ring-like lattice structure of the casein supramolecule observed in native casein micelles was still apparent.

6.9.5 Addition of Ethanol

In the presence of ethanol, the milk protein system undergoes destabilization (Horne 1992). This had been considered a function of alcohol reducing the dielectric constant causing a collapse of the brush-like C-terminal region of κ-casein on the casein supramolecule periphery. With the open interlocked lattice structure proposed by McMahon and Oommen (2008), this change in dielectric constant would be experienced by all proteins in the supramolecule. This would initiate a global contraction and rearrangement causing a collapse and partial dissociation of the casein supramolecules (Fig. 6.14). Horne and Davidson (1987) reported that the dissociated particles in a 1:1 trifluoroethanol/milk mixture were approximately the same hydrodynamic size as in native milk, but were dissimilar in molecular weight and sedimentation properties. Contrary to this explanation, O'Connell et al. (2001a), using confo-

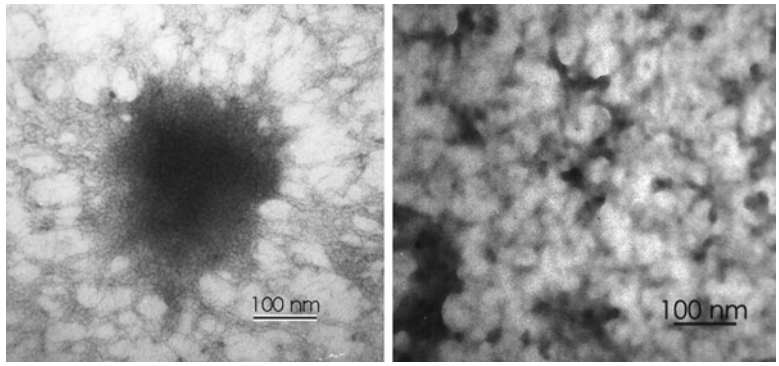

Fig. 6.14 Casein supramolecules and other proteins after mixing milk at 20°C 1:1 with ethanol (*left*) and after heating the mixture to 70°C (*right*) and imaged as in Fig. 6.2. Reprinted from Oommen (2004)

cal laser-scattering microscopy, observed that ethanol-modified casein supramolecules were smaller than native casein micelles. The presence of interconnected particles or fewer large aggregates that are in the process of dissociation might account for these differences. As seen in Fig. 6.14, the dissociated casein supramolecules appear to remain interconnected and have a large amount of void volume that can contribute to greater hydrodynamic radius while maintaining low molecular weight and consequently low sedimentation.

If a milk-ethanol mixture is heated, the casein supramolecules further dissociate (Zadow 1993; O'Connell et al., 2001a) into smaller compact particles and form aggregates (Fig. 6.14). Unlike independent particles, they were interconnected and could be considered as part of a larger aggregate. The nuclear magnetic spectra of both urea (6 mol/L)-treated milk and milk heated to 70°C in the presence of ethanol have been shown to be similar (O'Connell et al., 2001b). Urea dissociates casein supramolecules by enhancing protein solubility and by inhibiting hydrophobic bonding, and presumably ethanol has a similar effect. The increase in repulsive forces between caseins and solvent quality may result in dissociation of casein supramolecules when heated in presence of ethanol. Horne and Davidson (1987) attributed this dissociation of casein supramolecules to high helix development in caseins in presence of alcohols. The loss of internal structure and compactness of the small aggregates imply rearrangement of individual monomeric caseins from their initial-interlocked structure within the casein.

6.10 Conclusion

The supramolecular structure of casein micelles can be modeled as an interlocked lattice in which both casein-calcium phosphate aggregates and casein polymer chains act together to maintain casein micelle integrity. This model suggests that stabilization of calcium phosphate nanoclusters by phosphoserine domains of α_{s1}-, α_{s2}-, and/or β-casein would orient their hydrophobic domains outward allowing interaction and binding to other casein molecules. Other interactions between the caseins, such as calcium bridging, could also occur and further stabilize the supramolecule. The combination of having an interlocked lattice structure and multiple interactions results in an open sponge-like colloidal supramolecule that is resistant to spatial changes and disintegration unless the chemical environment is changed. The occluded spaces within the supramolecule matrix structure would be occupied by the serum phase of milk comprising water along with dissolved lactose, ions, and other soluble substances. Having open channels throughout the supramolecule means that virtually every casein molecule

is exposed to water molecules and their conformational shape will be influenced by the combined interactions with other protein, with calcium phosphate, with the water, and with related entropy considerations. A distinguishing feature of this interlocked lattice model is that any change in temperature or chemical environment will exert a global change throughout the supramolecule. For example, the observed decrease in particle size observed when alcohol is added to milk can be better explained by a global contraction of the supramolecule lattice structure rather than just collapse of a surface hairy layer. In this sense, the casein micelle itself can be considered rheomorphic as well as the individual casein molecules.

Hydrophobic interactions between caseins surrounding a calcium phosphate nanocluster would prevent complete dissociation of casein micelles when the calcium phosphate nanoclusters are solubilized. Likewise, calcium bridging and other electrostatic interactions between caseins would prevent dissociation of the casein micelles into casein-calcium phosphate nanocluster aggregates when milk is cooled, or urea is added to milk, and hydrophobic interactions are reduced. The appearance of both polymer chains and small aggregate particles during milk synthesis would also be expected based on this interlocked lattice model of casein micelles.

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β-Lactoglobulin

7

L. Sawyer

7.1 Introduction

The lipocalin family to which β-lactoglobulin, β-Lg, belongs has been expanding rapidly over the past couple of decades and now comprises at least 40 examples, widely spread throughout the biosphere (Åkerström *et al.*, 2006; Grzyb *et al.*, 2006), probably indicative of a bacterial origin (Sanchez *et al.*, 2006). The family has low sequence identity, generally less than 25%, but have a well-conserved tertiary structure comprising an antiparallel β-barrel or calyx. Mostly, members have a subunit molecular weight of 18–20 kDa, but several domains of larger proteins have also been found to adopt the lipocalin fold, some have enzymic activity and several, like insecticyanin and crustacyanin, bind chromophores (Table 7.1). However, the functions of quite a number are still, at best, ill-defined. Many seem to bind and transport a hydrophobic or labile small molecule, and some members appear to have been identified principally as allergens, including *Bos* d 2 which, although a bovine lipocalin, is quite distinct from β-Lg (Rouvinen *et al.*, 1999).

A protein sequence signature for the lipocalins is provided in the PRINTS database (Attwood *et al.*, 2003) and is an improvement on earlier versions (Pervaiz and Brew, 1985; Sawyer, 1987; North, 1991; Flower, 1996). The structurally conserved regions are highlighted in the lactoglobulin sequence comparison shown in a later section. North (1989, 1991) noticed that the conserved sequences are grouped at the base of the calyx furthest from its entrance such that a receptor recognition function is implied—a transporter needs to recognise its destination and might also need to signal whether it is carrying a ligand. The relatedness of the lipocalins is further supported by the similarity in gene sequences (Ali and Clark, 1988; Salier, 2000; Simpson and Nicholas, 2002; Sanchez *et al.*, 2006). The function of β-Lg in relationship to its being a lipocalin will be discussed after considering its molecular properties.

In the β-Lg chapter in the third edition of *Advanced Protein Chemistry* (Sawyer, 2003), the review covered the literature to the end of 2000. In the decade that has followed, there has been a significant body of work on the protein, further defining its structure, properties and increasingly, its applications in the general area of food and nutrition. Roughly one paper a day has been added covering both pure (i.e. properties and behaviour of β-Lg in its own right) and applied aspects, and it is the aim of this chapter to deal mainly with the molecular properties of the protein. The more applied aspects are covered elsewhere (e.g. Thompson *et al.*, 2009; see also Volume 1B).

β-Lg is the major whey protein secreted in the milk of ruminants like the cow or sheep. It is also found in the milk of monogastrics like the pig,

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Table 7.1 Selected members of the lipocalin family showing their wide distributiona

Protein	Source[b]	Amino acids	Location[b]	Ligand[c]	Function	PDB[d] entry	References
β-Lactoglobulin, *Bos* d 5 allergen	Many mammals	162	Milk	Fatty acids, vitamins A, D?	Transport/ transfer?	1BEB, 1EXS	Brownlow *et al.* (1997), Hoedemaeker *et al.* (2002)
Glycodelin	Human, baboon	162	Amniotic fluid	Retinol	Differentiation?	–	Seppala *et al.* (2009)
Retinol-binding protein[e]	Mammals, chicken	183	Blood serum	Retinol	Transport	1JYD	Cowan *et al.* (1990)
Apolipoprotein D	Human	169	Serum, gross breast cystic disease	Progesterone	Acute phase protein	2HZQ	Eichinger *et al.* (2007)
Complement protein C8γ	Human	182	Serum	Part of membrane attack complex	Immune system	1LF7	Ortlund *et al.* (2002)
α1-Acid glycoprotein, orosomucoid	Human	192	Serum		Acute phase protein	3KQ0	Schonfeld *et al.* (2008)
Neutrophil gelatinase-associated lipocalin, LCN2, siderocalin	Human	179	Serum, uterine secretion	Saccharides	Anti-bacterial, acute phase protein	1DFV	Holmes *et al.* (2005)
Tear lipocalin, von Ebner's gland protein	Human	162	Tears, saliva	Fatty acids?	Transport?	1XKI	Breustedt *et al.* (2005)
Odorant-binding protein	Cow, pig	159	Nasal mucous	Odorants	Transduction?	1DZK	Vincent *et al.* (2001)
Major urinary protein (MUP)	Mouse	162	Urine	2-*sec*-Butyl-4,5-dihydrothiazole	Marking?	3KFF	Perez-Miller *et al.* (2010)
Prostaglandin D synthase	Rat, human	168	Brain, cerebrospinal fluid	Prostaglandin H_2	PGD_2 synthesis	2CZT, 3O22	Kumasaka *et al.* (2009), Zhou *et al.* (Unpublished)
Bos d 2 lipocalin allergen	Cow	156	Sweat			1BJ7	Rouvinen *et al.* (1999)
α-Crustacyanin, C1 subunit	Lobster	181	Carapace	Astaxanthin	Camouflage?	1I4U	Gordon *et al.* (2001)
Nitrophorin	Tick	170	Saliva	Histamine/NO	Vasodilation	1NP1	Weichsel *et al.* (1998)
Bilin-binding protein	Butterfly	173	Epidermis	Biliverdin IX_γ	Camouflage	1BBP	Huber *et al.* (1987)
Domain of violaxanthin de-epoxidase	*Arabidopsis thaliana*	185	Thylakoid lumen	Violaxanthin	Zeaxanthin synthesis	3CQN	Arnoux *et al.* (2009)
Bacterial lipocalin, BLC	Bacteria	168	Membrane	Vaccenic acid	Stress response	3MBT	Schiefner *et al.* (2010)

The PRINTS lipocalin signature (Attwood *et al.*, 2003) identifies three structurally conserved regions beginning at residues 13, 94 and 121 in the β-Lg numbering

[a]A special issue of *Biochimica et Biophysica Acta*, **1482(2)** (2000) is devoted to the lipocalins. See also Åkerström *et al.* (2006)

[b]Source and location from which the protein can be derived. The distribution is generally more widespread

[c]Many lipocalins bind a variety of ligands, and often the physiological ligand is unknown

[d]Code for the coordinate set in the Protein Data Bank: http://www.ebi.ac.uk

[e]The cellular RBP, like the related fatty acid-binding protein, is a distinct protein with a 10-stranded barrel made up of fewer amino acids belonging to the wider calycin family

horse, dog, cat and in marsupials, but it is absent from the milk of humans, lagomorphs and rodents. Bovine β-Lg can be isolated readily, and since its isolation from milk by Palmer (1934), it has been, and still is, used extensively as a convenient small protein on which to try out new techniques, both experimental and theoretical. As well as Sawyer (2003), a number of specific reviews on the properties of β-Lg have been published over the years (e.g. Tilley, 1960; Townend *et al.*, 1969; McKenzie, 1971; Hambling *et al.*, 1992; Qin *et al.*, 1998b; Kontopidis *et al.*, 2004), together with several others on milk proteins in general, which contain substantial sections on the properties of the protein (e.g. McKenzie, 1967; Lyster, 1972; Thompson and Farrell, 1974; Jenness, 1979, 1985; Swaisgood, 1982; Kinsella and Whitehead, 1989; Fox, 1995; Farrell *et al.*, 2004; Edwards *et al.*, 2009).

Binding studies carried out on the bovine protein in vitro have shown that it can bind a variety of ligands, most of which are small, hydrophobic molecules like fatty acids or retinol. β-Lg undergoes several conformational changes between pH 2 and pH 9, possibly the most important of which, the N↔R, or Tanford, transition, occurs in the physiological pH range. The ruminant protein is a dimer under physiological conditions, whereas the β-Lg from other species appears to be monomeric. However, now that the crystal structures of β-Lg at several pH values have become available, a detailed molecular explanation of most of the solution properties is available (Qin *et al.*, 1998a; Sakurai *et al.*, 2009).

This chapter will expand upon the above outline to discuss the structure, molecular properties and possible function of the protein in some detail, covering the salient literature from the past eight decades. Inevitably, it can provide only an overview, coloured by the individual prejudices of the author.

7.2 Biosynthesis and Secretion

Major lactoproteins, including β-Lg, are biosynthesised within the secretory epithelial cells of the mammary gland under endocrine control from about mid-pregnancy (Simpson and Nicholas, 2002; see Chap. 14). The β-Lg gene comprises seven exons, the last of which is not translated in the mature protein. mRNA coding for β-Lg which is specific to the mammary tissue is translated to yield a 180 amino acid pre-β-Lg, whose signal peptides, some of which are shown in Fig. 7.1, contain highly conserved, largely hydrophobic amino acids (The Uniprot Consortium, 2008).

```
Cow         MKCLLLALAL--TCGAQA-
Buffalo     MKCLLLALGLALACAAQA-
Sheep       MKCLLLALGLALACGVQA-
Pig         MRCLLLTLGLALLCGVQA-
Horse I     MKCLLLALGLALMCGIQA-
Baboon      MQCLLLTLGVALICGVWA-
Wallaby     MKFLLLTVGLALIGAIQA-
Platypus    MKVLLLSIGLALVCAIQA-
             *. *** ::***  :: *
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Fig. 7.1 The signal peptides of several β-lactoglobulins (β-Lg) showing the high degree of conservation across the widely diverse species. Identities are shown as *asterisks*, near similarity as *colon* and more distant similarity as *period*

After removal of the signal peptide (Fig. 7.1), the mature protein undergoes disulphide bridge formation, within the rough endoplasmic reticulum. Transport to the Golgi and incorporation into secretory vesicles precede secretion into the lumen, where β-Lg accumulates in the milk before removal by suckling.

The promoter region of the β-Lg gene not only directs expression specifically to the mammary gland but also promotes high levels of expression. Consequently, transgenic mice have expressed in their milk native (Simons *et al.*, 1987) or modified (McClenaghan *et al.*, 1999) β-Lg from sheep, goat (Ibanez *et al.*, 1997) and cow (Bawden *et al.*, 1994; Hyttinen *et al.*, 1998). Further, studies on the promoter region have identified the minimum necessary for expression (Whitelaw *et al.*, 1992) as well as binding sites for, and the differing effects of, various promoters (e.g. Rosen *et al.*, 1999; Pena and Whitelaw, 2005; Braunschweig, 2007; Kotresh *et al.*, 2009; Fraser *et al.*, 2009).

Developments in mammary cell lines (German and Barash, 2002), PCR-RFLP (e.g. Caroli *et al.*, 2009), microarray technology (Chessa *et al.*, 2007) and of course genome sequences (Hubbard *et al.*, 2009; Lemay *et al.*, 2009; Elsik *et al.*, 2009) have led to more detailed studies of milk biosynthesis in general and that of β-Lg in particular, not only in bovine, ovine and caprine species but also in horse (Uniacke-Lowe *et al.*, 2010), donkey (Guo *et al.*, 2007) and the less economically important marsupial (Joss *et al.*, 2009) and fur seal (Cane *et al.*, 2005) milks. For example, Reichenstein *et al.* (2005) investigated a novel regulatory element in the ovine β-Lg promoter using a β-Lg-luciferase construct. The ability to detect polymorphisms efficiently has revolutionised the study of milk production trait loci (e.g. Caroli *et al.*, 2009) and the comparative biology of milk proteins (Simpson and Nicholas, 2002; Lemay *et al.*, 2009; Elsik *et al.*, 2009). Such studies have highlighted a number of non-coding mutations within the β-Lg gene that appear to be responsible for varying levels of protein expression (Ganai *et al.*, 2009) which in turn affect the commercial aspects of the milk production.

Although bovine and ovine β-Lgs were over-expressed in *Escherichia coli*, *Lactobacillus casei*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* as the near-native protein (Batt *et al.*, 1990; Rocha *et al.*, 1996; Hazebrouck *et al.*, 2007) or as a fusion protein (Ariyaratne *et al.*, 2002), over-expression at levels in excess of 100 mg β-Lg/L of culture supernatant was achieved only in *Pichia pastoris* (Kim *et al.*, 1997). Recently, however, Invernizzi *et al.* (2008) have achieved expression levels around 100 mg/L of soluble, secreted β-Lg in *E. coli*, but mutation of Cys_{121} led to insolubility. Ponniah *et al.* (2010), on the other hand, have reported overproduction of soluble bovine β-Lgs A and B in *E. coli* using a coding sequence optimised for the bacterium and co-expressing a disulphide isomerase. This system seems capable of producing mutated forms of the protein in quantities around 10 mg/L, labelled if required for NMR studies. Heterologous expression of both porcine (Invernizzi *et al.*, 2004) and equine (Kobayashi *et al.*, 2000) β-Lgs has also been reported. Site-directed mutagenesis of recombinant protein provides a powerful method for modifying the properties, which, for milk proteins, is providing a route to improved functionalities (Batt *et al.*, 1994; Whitelaw, 1999; see Chap. 16).

7.3 Distribution

The composition of milk varies with time since parturition, with species and also with season, the last presumably related to dietary habit. Qualitative and quantitative methods for the separation of whey proteins, useful for detecting the presence of β-Lg, have been reported by, *inter alia*, Davies (1974), Strange *et al.* (1992) and Otte *et al.* (1994). However, neither electrophoretic mobility nor polyclonal antibody cross-reactivity alone should be taken as proof of the presence of β-Lg (Bertino *et al.*, 1996; Conti *et al.*, 2000). During the past two decades, restriction fragment length polymorphism (Lien *et al.*, 1990), and the polymerase chain reaction (PCR) have been used to investigate the distribution of β-Lg (e.g. Jadot *et al.*, 1992; Prinzenberg and Erhardt, 1999). Isoelectric focussing, 2D and capillary electrophoresis have also been used successfully (Paterson *et al.*, 1995; Veledo *et al.*, 2005), more recently supplemented by mass spectrometry and microarray technologies (Chessa *et al.*, 2007).

Since the initial preparation of β-Lg from bovine (*Bos taurus*) milk (Palmer, 1934), dimeric β-Lg has been isolated from the milk of a number of other ruminants, and monomeric β-Lg has been purified from the milk of several nonruminant livestock species (Table 7.2). β-Lg has also been detected in milk of other species, but the state of its association in these cases is uncertain. For instance, although McKenzie *et al.* (1983) suggest that kangaroo β-Lg has a monomeric structure, later electrophoretic evidence implies that wallaby β-Lg exists as monomers, dimers and tetramers (Woodlee *et al.*, 1993).

While the milk of ruminants contains the β-Lg from a single gene, which may exist in distinct allelic forms, the milk from dog, dolphin, cat,

Table 7.2 Distribution of β-lactoglobulin in the milk of various species

A	B	C	D	E
Cow (*Bos taurus, B. javanicus, B. grunniens, B. indicus*)	✓✓	+[a]	Di[b,c]	1.8–5.0
Buffalo (*Bubalus arnee, B. bubalis*)	✓✓	+a	?Di	
Bison (*Bison bison*)	✓✓	+[a]	?Di[*]	
Musk ox (*Ovibos moschatus*)	✓	+[a]	?Di[*]	
Eland (*Taurotragus oryx*)	✓	+[a]	?Di[*]	
Goat (*Capra hircus*)	✓✓	+	?Di	1.4
Sheep (*Ovis aries, O. ammon musimon*)	✓✓	+	Di[d,e]	2.8
Red deer (*Cervus elaphus* L.)	✓	+[a]	?Di[*]	2.8–3.0
European elk (*Alces alces* L.)	✓	+[a]	?Di[*]	
Reindeer (*Rangifer tarandus* L.)	✓✓	+[a]	Di	
White-tailed deer (*Odocoileus virginianus*)	✓	+[a]	?Di[*]	
Fallow deer (*Dama dama*)	✓	+[a]	?Di[*]	
Caribou (*Rangifer arcticus*)	✓	+[a]	?Di[*]	
Giraffe (*Giraffa camelopardalis*)	✓	+[a]	?Di[*]	
Okapi (*Okapia johnstoni*)	✓	+[a]	?Di[*]	
Pronghorn antelope (*Antilocapra americana*)	✓	+[a]	?Di[*]	
Giant panda (*Ailuropoda melanoleuca*)	✓	Nm	?Mono[f]	
Bears (*Ursus americanus, U. maritimus, U. arctos horribilis, U. arctos yesoensis, U. arctos middendorffi, U. malayanus*)	✓	Nm	?Mono[g,h]	
Peccary (*Pecari tajacu*)	✓	–[*a]	Mono	
Pig (*Sus scrofa domestica*)	✓✓	±[i]	Mono	0.6
Horses (*Equus caballus, E. quagga, E. asinus*)	✓✓	±[i,j]	Mono[k]	
Rhinoceros (*Diceros bicornis*)	✓	+[a]	Mono[*]	
Rhinoceros (*Rhinoceros unicornis*)		Nm	Mono[*l]	2.3
Fur seals (*Callorhinus ursinus, Arctocephalus gazella, A. pusillus doriferus, A. tropicalis*)	✓✓	Nm	Mono[m]	
Dolphin (*Tursiops truncatus*)	✓✓	Nm	Mono[n]	16.2
Manatee (*Trichechus manatus latirostris*)	✓✓	Nm	Mono[n]	14.1
Dog (beagle) (*Canis familiaris*)	✓✓	Nm	Mono[n]	10.1
Cat (*Felis catus*)	✓✓	–[o]	?Mono[*]	
Grey kangaroo (*Macropus giganteus, M. rufus, M. eugenii*)	✓✓	Nm	Mono[*p]	
Echidna (*Tachyglossus aculeatus*)	✓✓	Nm	Mono[q]	
Brush tail possum (*Trichosurus vulpecula*)	✓✓	Nm	Mono	
Platypus (*Ornithorhynchus anatinus*)	✓✓	Nm	Mono[r]	
Yellow baboon (*Papio hamadryas*)	✓✓	Nm	?Mono	
Macaque (*Macaca fascicularis*)	✓✓	Nm	?Mono	
Human (*Homo sapiens*)		±[s]	None[*t]	<0.1118
Rabbit (*Oryctolagus cuniculus*)		–[a]	None	
Camel (*Camelus dromedarius*)		±[u]	?None	
Llama (*Lama glama* L.)		–[v]	None[v]	
Mouse (*Mus musculus*)		–	None[w]	0
Rat (*Rattus norvegicus*)		Nm	None[x]	0
Guinea pig (*Cavia porcellus*)		Nm	None[y]	0

Column A Species

Column B Presence of β-lactoglobulin

✓, Cross-reactivity or other information

✓✓, Some sequence information available

(continued)

Table 7.2 (continued)

Column C	Cross-reaction to anti-bovine antisera
	+, BLG detected by anti-bovine BLG antisera
	–, BLG not detected by anti-bovine BLG antisera
	±, BLG detected by anti-bovine BLG antisera, but only at higher titres
	N cross-reactivity to anti-bovine BLG antisera not measured
Column D	State of association
	Di dimeric BLG detected
	Mono monomeric BLG detected
	?Mono, ?Di BLG present, but its state of association unknown
	None no BLG present
	?None probably absent
	*Cross-reactivity to anti-bovine BLG antisera is assumed to indicate a dimeric form of the protein. In the case of human, the cross reactivity is an artefact
Column E	Quantity (mg/mL)

[a]Lyster *et al.* (1966); [b]Bull and Currie (1946); [c]Bell *et al.* (1981a, c); [d]Bell and McKenzie (1967b); [e]Godovac-Zimmermann *et al.* (1987); [f]Hudson *et al.* (1984); [g]Ando *et al.* (1979); [h]Jenness *et al.* (1972); [i]Liberatori *et al.* (1979a); [j]Bell *et al.* (1981c); [k]Godovac-Zimmermann *et al.* (1988); [l]Nath *et al.* (1993); [m]Cane *et al.* (2005); [n]Pervaiz and Brew (1986); [o]Halliday *et al.* (1991); [p]McKenzie *et al.* (1983); [q]Teahan *et al.* (1991); [r]Warren *et al.* (2008); [s]Liberatori *et al.* (1979b); [t]Bell and McKenzie (1964); [u]Liberatori *et al.* (1979c); [v]Fernandez and Oliver (1988); [w]Simons *et al.* (1987); [x]Hennighausen and Sippel (1982); [y]Brew and Campbell (1967)

horse and marsupials contains the product of two, or in some cases three, distinct genes (Collet and Joseph, 1995; Piotte *et al.*, 1998). While dogs, horses and donkeys, and cats express two or three distinct forms of β-Lg, marsupials produce a β-Lg and a late lactation protein more closely related to odorant-binding protein (Flower, 1996). Possum also produces a third lipocalin that is most like the major urinary proteins from rat and mouse (Piotte *et al.*, 1998; Watson *et al.*, 2007). β-Lg II of the cat, horse and donkey appears to be most closely related to the β-Lg pseudogenes (Piotte *et al.*, 1998; Pena *et al.*, 1999) identified in cow (Passey and MacKinlay, 1995) and goat (Folch *et al.*, 1996).

β-Lg is absent from the milk of the *Camelidae* (Kappeler *et al.*, 2003; Zhang *et al.*, 2005). β-Lg is also absent from rodent and lagomorph milks. The lack of hybridisation between cDNA from a rat mammary library and cDNA from sheep β-Lg (Simons *et al.*, 1987) preceded the whole genome studies of rat and mouse that confirmed the observation (Hubbard *et al.*, 2009). Similarly, β-Lg is absent from human and chimpanzee milk. Perhaps surprisingly, however, some primates do produce β-Lg: the macaque (*Macaca fascicularis* and *Macaca mulatta*—Azuma and Yamauchi, 1991; Kunz and Lönnerdal, 1994) and the yellow baboon (*Papio hamadryas*, Hall *et al.*, 2001), in which three alleles have been identified. As more complete mammalian genomes become available, a more detailed distribution will emerge, perhaps allowing a clear functional assignment to the protein. Of particular interest in this regard is that of the orangutan (*Pongo* spp.) whose relationship to humans is between that of the chimpanzee and the baboon (Hubbard *et al.*, 2009), but in which at this stage, only the lipocalin glycodelin appears to have been identified.

7.4 Isolation

The isolation of β-Lg from milk is a simple procedure, involving just four stages: removal of fat, removal of the caseins, fractionation of the whey proteins and the final purification of β-Lg. Since each stage can be carried out in a number of ways, various protocols are available and others continue to be published.

The original isolation by Palmer (1934) was superseded by that of Aschaffenburg and Drewry

(1957) and scaled up by Mailliart and Ribadeau-Dumas (1988). Armstrong *et al.* (1967) replaced the potentially harsh pH treatment by precipitation at pH 3.5, and Monaco *et al.* (1987) used DEAE-cellulose chromatography to keep the pH around 6.5 throughout. More recent methods, ideally suited for bulk separations, rely on gel filtration, membrane filtration and ultrafiltration (e.g. Brans *et al.*, 2004; Saufi and Fee, 2009), ion exchange and hydrophobic interaction chromatography (e.g. Kristiansen *et al.*, 1998; Lozano *et al.*, 2008). Affinity chromatography and exploiting β-Lg complexes of retinol (Heddleson *et al.*, 1997) or retinal (Vyas *et al.*, 2002) have been described also, the latter finding that a fluidised bed produced the best results. Presumably because other methods work well, little work on ethanol fractionation of β-Lg has been reported since that of Bain and Deutsch (1948) on cow and goat milk which suggested the protein had denatured. Dimethyl sulphoxide can also be used although there appears to be little advantage (Arakawa *et al.*, 2007).

Other dimeric β-Lgs can be obtained by these or similar methods. Thus, first reports exist for the isolation of β-Lg from yak (Grosclaude *et al.*, 1976; Ochirkhuyag *et al.*, 1998), red deer (McDougall and Stewart, 1976), reindeer (Heikura *et al.*, 2005), water buffalo (Kolde *et al.*, 1981), sheep (Maubois *et al.*, 1965) and goat (Kalan and Basch, 1969). Both goat (Préaux *et al.*, 1979) and sheep (Godovac-Zimmermann *et al.*, 1987) β-Lgs have also been isolated by gel filtration (cf., Davies, 1974; Strange *et al.*, 1992).

Nonruminant β-Lg can be isolated with equal ease using procedures similar to those already described. Pig β-Lg has been purified by Jones and Kalan (1971), Ugolini *et al.* (2001) and Kessler and Brew (1970), the latter method being adapted for the monomeric β-Lgs from dolphin, manatee, beagle (Pervaiz and Brew, 1986) and horse (Godovac-Zimmermann *et al.*, 1985; Ikeguchi *et al.*, 1997).

Heterologous expression and purification of β-Lg from several species has also been described both from bacteria (Ariyaratne *et al.*, 2002; Ponniah *et al.*, 2010) and yeast (Kim *et al.*, 1997; Denton *et al.*, 1998; Invernizzi *et al.*, 2004). The methods used are generally ion exchange followed by gel filtration, simplified in some cases by expression of a fusion protein followed by affinity chromatograph (e.g. Ariyaratne *et al.*, 2002).

The amount of β-Lg obtained by the various methods depends upon both the procedure used and the quantity of β-Lg in the initial milk, which is known to vary with species, season and time since parturition. The quantity of β-Lg isolated from the milk of a few species is given in Table 7.2.

7.5 Genetic Variants and Primary Structure

Over the past two decades, there has been significant interest in the genetic variability of milk proteins with respect to production and processing properties, including the effect of the principal β-Lg variants (Hill *et al.*, 1996). The effects are more thoroughly reviewed elsewhere (e.g. Caroli *et al.*, 2009; Ganai *et al.*, 2009).

Although Li (1946) and Polis *et al.* (1950) separated two components of β-Lg, it was Aschaffenburg and Drewry (1955, 1957) who showed that the two components in bovine milk were genetically determined. Genetic variants of β-Lg also exist in other ruminant species, while distinct genes appear in other mammals (Piotte *et al.*, 1998; Pena *et al.*, 1999). Ion exchange was used to separate the two most common bovine genetic variants, A and B (Piez *et al.*, 1961), but many other genetic variants of bovine β-Lg have now been identified (Farrell *et al.*, 2004). Other convenient phenotyping methods include gel electrophoresis of whole milk (Davies, 1974; Lowe *et al.*, 1995), capillary electrophoresis (Paterson *et al.*, 1995; de Frutos *et al.*, 1997; Schopen *et al.*, 2009), isoelectric focussing (Godovac-Zimmermann *et al.*, 1990; Fernandez-Espla *et al.*, 1993; Dorji *et al.*, 2010), HPLC (Presnell *et al.*, 1990; Miranda *et al.*, 2004), molecular biological techniques (Jadot *et al.*, 1992; Schlee *et al.*, 1993; Feligini *et al.*, 1998; Rachagani *et al.*, 2006; Caroli *et al.*, 2009) and mass spectrometry (Criscione *et al.*, 2009).

The first correct amino acid sequence of bovine β-Lg was reported by Braunitzer *et al.* (1972), and the position of one disulphide, 66–160, was identified unambiguously (Préaux

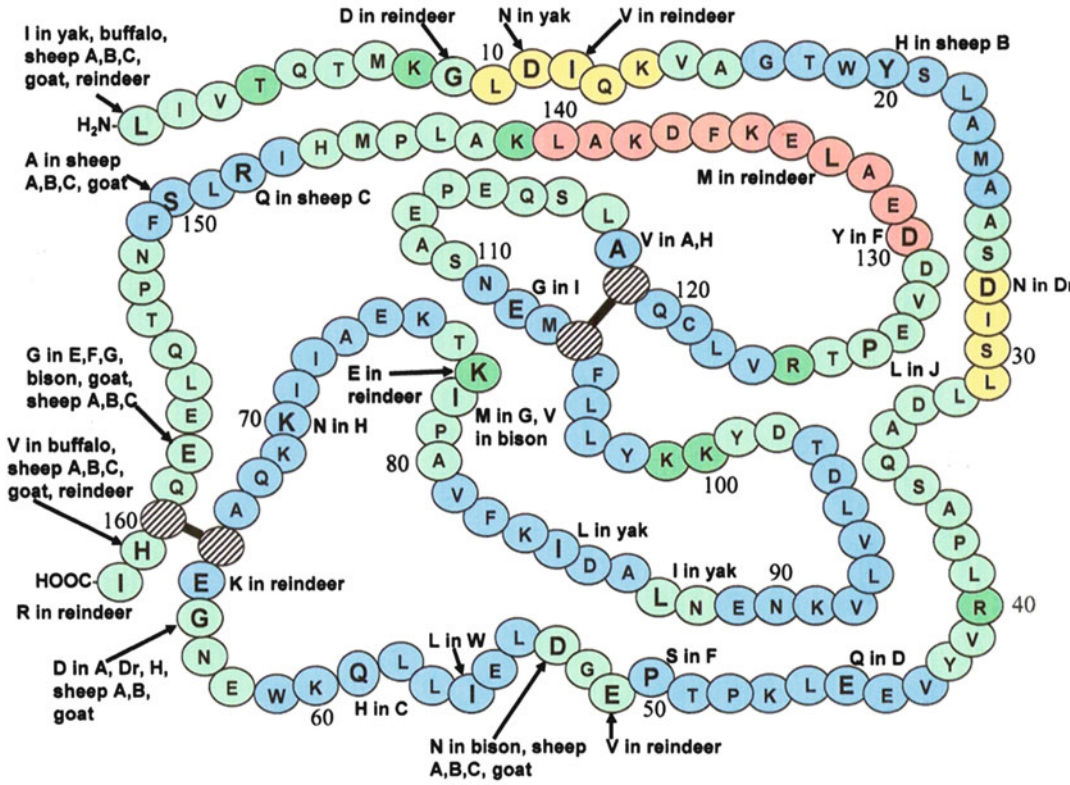

Fig. 7.2 Amino acid sequence variation within ruminant β-lactoglobulins relative to bovine genetic variant B. The sequence is represented in *single-letter* notation with residues in *larger font* indicating positions of genetic variation. Those variations referring to the domestic cow only have the variant letter: *N in Dr* refers to the Droughtmaster breed in which residue Asp_{28} is changed to Asn. Other species are referred to explicitly: *I in yak* refers to Leu_{87} in cow becoming Ile in the yak. The two disulphide bridges between residues 66–160 and 106–119 are shown with *black hatching*. *Yellow* indicates regions of 3_{10} helix, *blue* shows β-strands and *brown* depicts α-helix. The figure is adapted from the one kindly provided by the Fonterra Research Centre, Palmerston North, New Zealand, with permission

and Lontie, 1972). The other disulphide, 106–119, with a free cysteine at position 121, remained uncertain until the crystal structure emerged (McKenzie and Shaw, 1972; Papiz *et al.*, 1986; Sawyer, 2003), the uncertainty eventually explained as urea-mediated disulphide interchange (Phelan and Malthouse, 1994).

Figure 7.2 presents the differences relative to β-Lg B of the ruminant species that are synthesised under the control of codominant alleles. β-Lg from a subset of Droughtmaster cattle, glycosylated at Asn_{28} (Bell *et al.*, 1981b) as it is in the related glycodelin, and minor truncated components found only in the milk of Romagnola cattle (Zappacosta *et al.*, 1998) are the only atypical phenotypes, although the truncation may not be of genetic origin. Interestingly, although polymorphisms have been detected in the coding regions of the goat gene, none leads to an amino acid change (Ballester *et al.*, 2005).

Many nonruminant β-Lgs have now been sequenced, and their sequences diverge considerably from that of bovine β-Lg B and from one another. Many of the substitutions observed cannot have arisen from single point mutations. As noted already, the milk of dolphin, dog, cat, donkey, horse and pig contains β-Lg from more than a single gene, and indeed ruminant pseudogenes have been identified that appear to reflect these other genes (see above). The complete sequences of the available monomeric β-Lgs, together with a representative ruminant sequence, are shown in Table 7.3. The data are

Table 7.3 Amino acid sequences of β-lactoglobulin for the nonruminant species relative to that of bovine β-lactoglobulin B

```
                    3 33333 AAAA AAAAA  333 3            BBBBBBBB    CCCCCCC
Wallaby      VENIRSKNDL GVEKFVGSWY LREAAKT--- -MEFSIPLFD MDIKEVNLTP EGNLELVLLE 56
Kangaroo     VENIRSKNDL GVEKFVGSWY LREAAKT--- -MEFSIPLFD MDIKEVNLTP EGNLELVLLE 56
Possum       IENIHSKEEL VVEKLIGPWY RVEEAKA--- -MEFSIPLFD MNIKEVNRTP EGNLELIVLE 56
Cow B        LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP EGDLEILLQK 60
Dolphin      VSVIRTMEDL DIQRVAGTWH SVAMAASDIS LLDTESAPLR VNVEELRPTP QGDLEIFLQK 60
Pig          VEVTPIMTEL DTQKVAGTWH TVAMAVSDVS LLDAKSSPLK AYVEGLKPTP EGDLEILLQK 60
Ass I        TNIPQTMQDL DLQEVAGKWH SVAMAASDIS LLDSEEAPLR VYIEKLRPTP EDNLEIILRE 60
Horse I      TNIPQTMQDL DLQEVAGKWH SVAMAASDIS LLDSESAPLR VYIEKLRPTP EDNLEIILRE 60
Dog          IVVPRTMEDL DLQKVAGTWH SMAMAASDIS LLDSETAPLR VYIQELRPTP QDNLEIVLRK 60
Dog III      IVIPRTMEDL DLQKVAGTWH SMAMAASDIS LLDSETAPLR VYIQELRPTP QDNLEIVLRK 60
Cat I        ATVPLTMDGL DLQKVAGMWH SMAMAASDIS LLDSETAPLR VYVQELRPTP RDNLEIILRK 60
Cat III      ATVPLTMDGL DLQKVAGTWH SMAMAASDIS LLDSEYAPLR VYVQELRPTP RDNLEIILRK 60
Ass II       TDIPQTMQDL DLQEVAGRWH SVAMVASDIS LLDSESAPLR VYVEELRPTP EGNLEIILRE 60
Horse II     TDIPQTMQDL DLQEVAGRWH SVAMVASDIS LLDSESVPLR VYVEELRPTP EGNLEIILRE 60
Cat II       ATLPPTMEDL DIRQVAGTWH SMAMAASDIS LLDSETAPLR VYVQELRPTP RDNLEIILRK 60
Seal         IVVPRTMEDL DLQKVAGTWH SMAMAASDIS LLDAKTAPLR VYVQELRPTP EGNLEIVLRK 60
Baboon       INSPQTMQDV ELPKLAGTWH SMAMAASDFS LLETKEAPLR IYISSLQPTP EGNLEIVLRR 60
Macaque      IDSPQTMQDV ELPKLAGTWH SMAMAASDFS LLETKEAPLR IYISSLQPTP EGNLEIALRR 60
Mac_Gly      TDMPQTKQNL ELPKLAGTWH SMAMATNNVS LMVMVKSALR VHVTSLWPTP EDHLEIVLHR 60
Glycodelin   MDIPQTKQDL ELPKLAGTWH SMAMATNNIS LMATLKAPLR VHITSLLPTP EDNLEIVLHR 60
                      :  :   .* *:        : * *:      :: .    :  **   .**: : :
Pseudo       AYIPQMAGDL DIRKVAGMWH TVAMAASNML LLDAESGPLR VYVEDLKPTP EGDLEILLQK 60
Goat pseudo  AHIPQTVEDL DIRKVARTRH PVAMAASNVL LLDTES---- VYVEELKPTP EGDLEILLQK 56
                      :  .   .: ::        :   *:      :: .    :  **   .**: : :
Platypus     -VADVSDKPI SFEKLAGPWH TILLATNDKE MIK-EDEKMK LLFKTVTPQN TKELIITMLK 58
                      :       : ::        :   ::      :: .    :       .* : : :


             C            DDDDDDDD DDD     EE EEEE  FF    FFFFFFF     GGGGGGGG
Wallaby      K---------- ADRCVEKKLL LKKTQKPTEF EIYISSESAS YTFSVMETDY DSYFLFCLYN 107
Kangaroo     K---------- TDRCVEKKLL LKKTKKPTEF EIYISSES-S YTFCVMETDY DSYFLFCLYN 106
Possum       Q---------- TDSCVEKKFL LKKTEKPAEF EIYIPSESAS YTLSVMETDY DNYILGCLEN 107
Cow_B        W---------E NGECAQKKII AEKTKIPAVF KIDALNEN-- -KVLVLDTDY KKYLLFCMEN 109
Dolphin      R---------D KNGCVKEKII A-KTEIPAVF KINFLNEN-- -KIFVLDSDY TNYLLFCMEN 108
Pig          R---------E NDKCAQEVLL AKKTDIPAVF KINALDEN-- -QLFLLDTDY DSHLLLCMEN 109
Ass I        G---------E NKGCAEKKIF AEKTESPAEF KINYLDED-- -TVFALDSDY KNYLFLCMKN 109
Horse I      G---------E NKGCAEKKIF AEKTESPAEF KINYLDED-- -TVFALDTDY KNYLFLCMKN 109
Dog          W---------E DGRCAEQKVL AEKTEVPAEF KINYVEEN-- -QIFLLDTDY DNYLFFCEMN 109
Dog III      W---------E DNRCVEKKVF AEKTELAAXF SINYVEEN-- -QIFLLDTDY DNYLFFCMEN 109
Cat I        W---------E DNRCVEKKVL AEKTECAAKF NINYLDEN-- -ELIVLDTDY ENYLFFCLEN 109
Cat III      W---------E QKRCVQKKIL AQKTELPAEF KISYLDEN-- -ELIVLDTDY ENYLFFCLEN 109
Ass II       G---------A NHVCVERNIV AQKTEDPAVF TVNYQGER-- -KISVLDTDY AHYMFFCVGP 109
Horse II     G---------A NHACVERNIV AQKTEDPAVF TVNYQGER-- -KISVLDTDY AHYMFFCVGP 109
Cat II       R---------E NHACIEGNIM AQRTEDPAVF MVDYQGEK-- -KISVLDTDY THYMFFCMEA 109
Seal         W---------E NSACVEGNIV AQKTEDPAVF TVDYQGQR-- -KISVLDTDY THYLFFCMEA 109
Baboon       WSQKQSPFRD SNQCIEEKII AEKTENPIEF KINYLDEN-- -RIYLFNTDG SKYLFLCLES 117
Macaque      WSQKQSPFRE SNQCIEEKII AEKTENPIEF KINYLDEN-- -RIYLFNTDG SKYLFLCLES 117
Mac_Glycod   W---------E NNSCVEKKVL GEKTENPKKF KINYMGAN-- -EAMLLDTDY DNFLFLCLTD 109
Glycodelin   W---------E NNSCVEKKVL GEKTENPKKF KINYTVAN-- -EATLLDTDY DNFLFLCLQD 109
                        *:   ..  .:*. .  *               .    :*    ::::*.
Pseudo       R---------E NHECVEKTLM AQKTEDPAVF TVNYHGER-- -KISVRDTDY SSYTIFCMEG 109
Goat_pseudo  R---------E NHECVEKTLM AQKTEDPTVF TVDYHGER-- -KISVLDTDY SSYMTFCKEG 105
                        *:   ..  .:*. .  *               .    :*    :: :*
Platypus     K---------E NHECTEYNLV IKQTEEPNKF RPVLSTDNEK DIIIFVDSDY LNYFLVVFQN 110
                        * :  ..  .:*. .  *               .    :*    :  :
```

(continued)

Table 7.3 (continued)

```
                        HHHHH       αααααααα αα      II III
Wallaby      ---ISDREKM ACAHYVRRIE -ENKGMNEFK KILRTLAMPY TVIEVRTR-- DMCHV----- 156
Kangaroo     ---ISDREKM ACAHYVRRIE -ENKGMNEFK KILRTLAMPY TVIEVRTR-- DMCHV----- 155
Possum       ---VNYREKM ACAHYERRIE -ENKGMEEFK KIVRTLTIPY TMIEAQTR-- EMCRV----- 156
Cow B        --SAEPEQSL ACQCLVRTPE VDDEALEKFD KALKALPMHI RLSFNPTQLE EQCHI----- 162
Dolphin      --TADPERSL TCQYLARTLQ VDDGVMEKFN KAIKPLPMHI RLSFSPTQLE EQCRV----- 161
Pig          --SASPEHSL VCQSLARTLE VDDQIREKFE DALKTLSVPM RI--LPAQLE EQCRV----- 160
Ass I        --AATPGQSL VCQYLARTQM VDEEIMEKFR RALQPLPGRV QIVPDLTRMA ERCRI----- 162
Horse I      --AATPGQSL VCQYLARTQM VDEEIMEKFR RALQPLPGRV QIVPDLTRMA ERCRI----- 162
Dog          --ADAPQQSL MCQCLARTLE VDNEVMEKFN RALKTLPVHM QLLN-PTQAE EQCLI----- 161
Dog III      --ANAPQQSL MCQCLARTLE VNNEVIGKFN RALKTLPVHM QLLN-PTQVE EQCLV----- 161
Cat I        --ADAPDQNL VCQCLTRTLK ADNEVMEKFD RALQTLPVHV RLFFDPTQVA EQCRI----- 162
Cat III      --ADAPGQNL VCQCLTRTLK ADNEVMEKFD RALQTLPVDV RLFFDPTQVA EQCRI----- 162
Ass II       C-LPSAEHGM VCQYLARTQK VDEEVMEKFS RALQPLPGHV QIIQDPSGGQ ERCGF----- 163
Horse II     P-LPSAEHGM VCQYLARTQK VDEEVMEKFS RALQPLPGRV QIVQDPSGGQ ERCGF----- 163
Cat II       P-APGTENGM MCQYLARTLK ADNEVMEKFD RALQTLPVHI RIILDLTQGK EQCRV----- 163
Seal         P-VPTAESGM MCQYLARTLK VNNEVMGKFN RALETLPVHM QIIPDLTQGK EQCHVV---- 164
Baboon       --TRR--QNL ACQYLARTLE VDDKVMAEFI SFLKTLPVHM QIFLDMTQAE EQCRV----- 168
Macaque      --TPR--QNL ACQYLARTLE VDDKVMAEFI SFLKTLPVHM QIFLDMTQAE EQCRV----- 168
Mac Gly      --TTTRIQCL MCQYLARVLV EDDEIMKGFI RAFRPLHKRL WYLLDLRKTE EPCHF----- 162
Glycodelin   --TTTPIQSM MCQYLARVLV EDDEIMQGFI RAFRPLPRHL WYLLDLKQME EPCRF----- 162
                        :  *: . *     ..  :  *    :  *   .             : *
Pseudo       D-AHR-EGSV MCQCLARTPE MDDEAMEKFA RALASLLEHV QMVLDLRQGA EQCHI----- 162
Goat pseudo  P-THTDEGSV MCQCLARTPE VDDEAMEKFA RALAFLSEDV QMVLDLRRGA GQCHV----- 159
                        :  *: . *     ..  :  *    :  *   .             . *
Platypus*    YNEELDREDT VVQCLSRTFD LTTEAEEKFN KVLKDYNISE ENVINLNNEK DKGGWLGAGK 170
                          : . *           *    :  :                 .
```

The sequences of β-Lg from nonruminant species; the bovine B variant is included as representative of the ruminant proteins. Bovine and caprine pseudogenes, the human and macaque glycodelins (Gcn) are also included. The sequences are divided every ten residues. The bold regions of the cow sequence represent the lipocalin motifs defined by PRINTS (Attwood *et al.*, 2003). The secondary structure observed for the lattice *X* form of bovine β-Lg is shown above the cow sequence: 3 = 3_{10}-helix; A,B,C, etc. = β-strand A,B,C, etc.; α = α-helix. Conserved residues are indicated if strictly conserved (*), similar (:) or broadly similar (.). The Swiss-Prot databank entries are cow—P02754; cow pseudogene (Passey and Mackinlay, 1995); goat pseudogene—Z47079; the author is indebted to Dr J. M. Folch for help with the translation; dolphin—B61590 (Pervaiz and Brew, 1987); pig—P04119; dog—P33685; dog III—P33686; cat I—P33687; cat III—P33688; donkey I—P13613; horse I—P02758; donkey II—P19647; horse II—P07380; cat II—P21664; baboon—AF021261; macaque (Hall *et al.*, 2001); glycodelin—P09466; macaque glycodelin—Q5BM07; wallaby—Q29614; kangaroo—P11944; possum—Q29146; platypus–F65×48

mostly retrieved from the Swiss-Prot databases (The Uniprot Consortium, 2008).

A number of partial and complete cDNA sequences (Willis *et al.*, 1982; Mercier *et al.*, 1985; Gaye *et al.*, 1986; Jamieson *et al.*, 1987; Ivanov *et al.*, 1988) were followed by complete gene sequences (Ali and Clark, 1988; Alexander *et al.*, 1989) that revealed the pattern of introns subsequently found to be consistent among the wider lipocalin family (Salier, 2000; Sanchez *et al.*, 2006).

Examination of the primary structures of β-Lg reveals no obviously repetitive or unusual stretches of sequence. There are, however, the structurally conserved regions indicative of a core member of the lipocalin family (Flower, 1996). Although the glycosylated β-Lg_{Dr} (Bell *et al.*, 1981b) is atypical of ruminant species, Batt and co-workers have produced a glycosylated bovine β-Lg by mimicking the glycosylation sites of the related lipocalin, glycodelin (Kalidas *et al.*, 2001), including the Asn_{28} of β-Lg_{Dr}. The nonruminant sequences show much lower sequence homology (typically 30–70% identity), in keeping with their being the product of separate genes (Sanchez *et al.*, 2006). In passing, it should be noted that β-Lg contains all 20 amino acids in relative amounts that make it valuable nutritionally.

7.6 Structure

Both macromolecular (X-ray) crystallography and NMR spectroscopy have been widely applied to β-Lg, and it is convenient to describe the molecular structure of β-Lg here as a basis for understanding the protein's properties. The need for suitable crystals for the X-ray technique is obviously a limitation, although as far as can be judged, the structures obtained are generally a fair reflection of the solution state of the protein. On the other hand, heteronuclear NMR spectroscopy that produces a structure in solution, generally requires a suitable over-expression system. Both techniques therefore have played an important role in our understanding of the structure and properties of β-Lg.

Although β-Lg was one of the first proteins to be subjected to X-ray analysis (see Hodgkin and Riley, 1968), and low-resolution work on salted out forms, lattices *X*, *Y* and *Z*, was summarised by Green *et al.* (1979), the first high-resolution structure of the triclinic X form of the cow protein (Brownlow *et al.*, 1997) corrected the threading errors in the medium resolution structure (Papiz *et al.*, 1986) and provided an independent view of the dimer. Structures of crystal forms with a monomer in the asymmetric unit require the molecular twofold rotation axis to be coincident with a crystallographic one (Bewley *et al.*, 1997; Qin *et al.*, 1998a, b). Detailed X-ray crystallographic studies on the native/recombinant protein *inter alia* have now been carried out on several crystal forms of bovine β-Lg (Table 7.4), as well as the pig and reindeer proteins, and it is with reference to these structures that we discuss the molecular properties of the protein.

In solution, the experimental values for α-helix, β-sheet and random coil content around 8%, 45% and 47%, respectively, are broadly similar to the values predicted from the sequence (see Sawyer, 2003). These values agree with what is observed in the crystal and NMR structures. However, most prediction methods indicate a significantly greater helical content than is

Table 7.4 Crystal structure data for native β-lactoglobulins

Resolution (Å)	Space group	Lattice code[a]	Z[b]	pH	β-Lg[c]	PDB code[d]	References
2.8	$B22_12$	*Y*	1	7.6	A		Papiz *et al.* (1986)
1.7	P1	*X*	2	6.5	AB	1beb	Brownlow *et al.* (1997)
1.8–2.0	$C222_1$	*Y*[e]	1	7.6	A, B, C		Bewley *et al.* (1997)
2.56	$P3_221$	*Z*	1	6.2	A	3blg	Qin *et al.* (1998a)
2.24	$P3_221$	*Z*	1	7.1	A	1bsy	Qin *et al.* (1998a)
2.49	$P3_221$	*Z*	1	8.2	A	2blg	Qin *et al.* (1998a)
2.2	$P3_221$	*Z*	1	7.1	B	1bsq	Qin *et al.* (1999)
2.4	$P3_221$	*Z*	1	3.2	Pig	1exs	Hoedemaeker *et al.* (2002)
2.0	$C222_1$	*Y*	1	7.9	A	1qg5	Oliveira *et al.* (2001)
1.95	$C222_1$	*Y*	1	7.9	B	1b8e	Oliveira *et al.* (2001)
3.0	$P2_12_12_1$	*U'*	4	5.2	A	2akq	Adams *et al.* (2006)
2.1	P1		8	6.5	Reindeer	1yup	Oksanen *et al.* (2006)
2.1	$C222_1$	*Y*	1	7.4	A	2q2m	Vijayalakshmi *et al.* (2008)
2.2	$P3_221$	*Z*	1	7.5	B	3npo	Loch *et al.* (2011)
2.0	$P6_5$		2	7.0	Gyuba[f]	3kza	Ohtomo *et al.* (2011)

[a]The lattice code is that assigned by Aschaffenburg *et al.* (1965)
[b]Z is the number of β-Lg monomers per asymmetric unit. $Z = 1$ means that the dimer has a strict crystallographic twofold rotation axis
[c]The β-Lg used cow unless otherwise stated. A, B, C refer to the cow β-Lg genetic variant
[d]The PDB code is that given to the atomic coordinates by the Protein Data Bank (http://www2.rcsb.org/pdb/home/home.do)
[e]This is the conventional setting of $B22_12$, originally designated as lattice *Y*
[f]Gyuba is a chaemera made from strands of bovine β-Lg and the loops of the equine protein

observed (Sawyer and Holt, 1993; Sakurai *et al.*, 2009) which has significant implications for the folding of the protein. The structure of the monomer consists of nine strands of antiparallel β-sheet, eight of which wrap round to create a flattened, conical barrel or calyx, closed at one end by Trp_{19}. Strand A bends through about 90° around residues 21–22 to allow it to form an antiparallel interaction with strand H, thereby completing the calyx. The calyx is approximately cylindrical with a volume of 315 $Å^3$ and a length of some 15 Å and walls that are hydrophobic. It has been suggested that the calyx is empty unless occupied by a ligand (Qvist *et al.*, 2008). A ninth strand, I, is on the outside, on the opposite side of strand A to strand H, and so is able to form part of the dimer interface which buries 570 $Å^2$ on each monomer. The interface involves antiparallel interactions of residues 146–150 with those of the other subunit, together with Asp_{33}, Ala_{34} and Arg_{40} in the large AB loop, the Asp-Arg forming an essential inter-subunit ion pair (Sakurai and Goto, 2002). There is a 3-turn α-helix on the outer surface of the calyx over strand H that is not in contact with its equivalent in the other subunit. The polypeptide chain between the β-strands includes two separate 3_{10}-turns and a γ-turn, conserved in all lipocalins that have the $–T_{97}DY_{99}–$ sequence. The fold of the monomer is shown in Fig. 7.3, which also shows the bovine dimer.

While the reindeer structure is essentially the same as that of the cow, the porcine structure (Fig. 7.4) differs in a number of respects (Hoedemaeker *et al.*, 2002). Most obviously, the dimerisation that occurs at low pH (cf. the cow protein) is quite distinct and involves a domain swap of the N-terminal 12 residues so that, for example, Glu_9 of subunit A binds to Thr_{142} of subunit B. The bulk of the polypeptide chain, however, follows the typical fold with β-strands A–I in a similar relationship to those of the bovine protein. The major helix, too, is similarly arranged. The dimer formed by the chaemeric 'Gyuba' protein closely resembles that of the cow and reindeer proteins since the core β-sheet has been retained, while the loop regions have been grafted from the equine protein (Ohtomo *et al.*, 2011; 'Gyuba' is from the Japanese words for cow and horse).

Several analyses show the differences between the A, B (and C) genetic variants (Bewley *et al.*, 1997; Qin *et al.*, 1999; Oliveira *et al.*, 2001). The A/B sequence changes are Asp64Gly and Val118Ala. The effects on the solution behaviour of these small changes are, however, significant (Townend *et al.*, 1964; Jakob and Puhan, 1992; Hill *et al.*, 1996; Manderson *et al.*, 1998, 1999a, b) and appear to arise largely from the Val/Ala change at 118 in strand H creating a cavity, and hence less favourable packing, in the core. The destabilisation of the B variant relative to the A has been estimated to be around 5 kJ/mol (Alexander and Pace, 1971; Qin *et al.*, 1999), which is expected for the loss of two methyl groups (Shortle *et al.*, 1990). Interestingly, a hydrogen–deuterium exchange study suggests that the A variant is more flexible than the B variant (Dong *et al.*, 1996). The Asp/Gly change occurs in the flexible external loop CD, and so its effect is less marked. In the C variant, the change Gln59His is at the end of the C strand causing a redistribution of side-chain interactions that affect the solution behaviour (Bewley *et al.*, 1997).

Fig. 7.3 (continued) rear of the picture is in the open position in this structure. The drawing was made using PyMOL (2008). (**b**) The dimer of β-Lg showing the interaction sites in *magenta*: the antiparallel arrangement of strands I together with residues in the AB loop, in particular Asp_{33} and Arg_{40}. Also shown is the buried carboxyl from Glu89 on the EF loop in *orange red*, which is in the closed position. Notice that the EF loop in the right-hand, *blue* subunit has a break where the electron density was poor. The drawing was made using PyMOL (2008). (**c**) The dimer interface of β-Lg is shown 'opened out': If the molecular twofold axis is perpendicular to the plane of the page, the *left-hand* part of the molecule has been rotated about 90° about a vertical axis, the *right hand* has been rotated similarly in the opposite direction. The *stick* representation of the main chain surrounded by a semitransparent surface is shown with the area surrounding the AB loop and the I strand indicating the contact surface which is also shown on the *right-hand* part of the figure. Notice that strand I and the AB loop, in particular the Asp33–Arg40 interaction, are the only points of contact between the monomers. The drawing was made using PyMOL (2008)

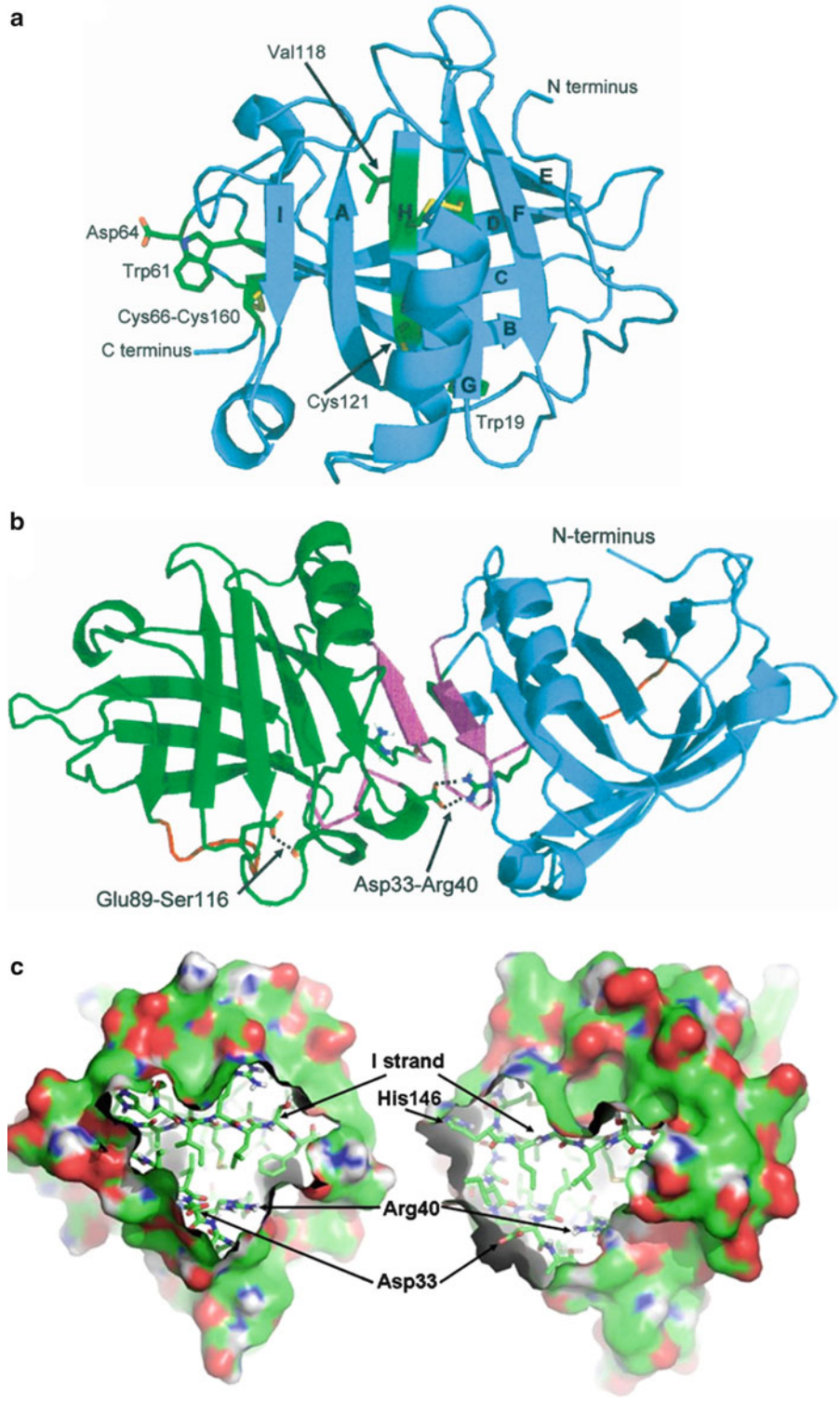

Fig. 7.3 (**a**) The monomer of β-lactoglobulin A viewed approximately down the molecular twofold axis. The Asp_{64} and Val_{118} residues are those changed to Gly and Ala, respectively, in the B variant. The disulphides 66–160 and 106–119 are shown together with the free Cys_{121}. Tryptophans 19 and 61 are also shown. The EF loop at the

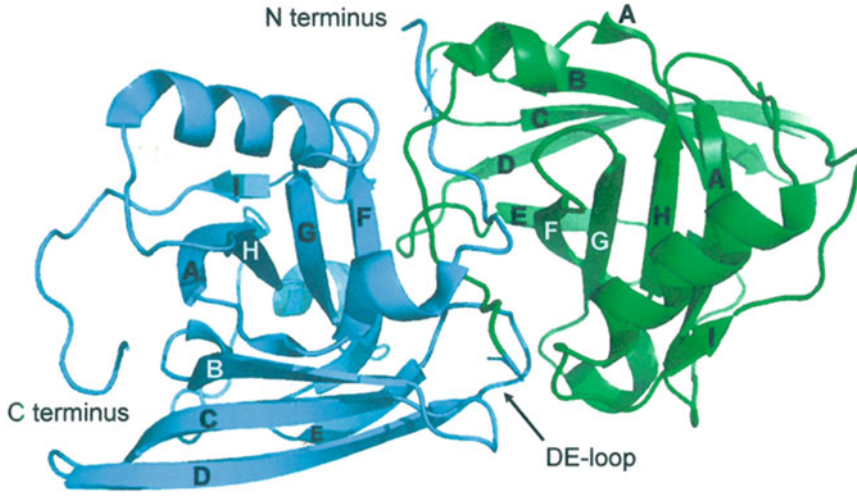

Fig. 7.4 The dimer of porcine β-Lg showing the quite distinct dimer formation from the ruminant protein. The I strands have no contacts shorter than 4 Å, and the two N-terminal regions are 'domain swapped', the *blue* residues interacting with the *green* subunit and vice versa. The drawing was made using PyMOL (2008)

Crystallographic analyses can provide some information about the mobility or flexibility of the protein main and side chains from the B (or temperature) factors: the larger the value, the less well defined, or the more mobile, the atom. With β-Lg, several sections of the polypeptide chain regularly appear to have high B factors, and the corresponding electron density is weak and indistinct. Comparison of the various crystal forms of the protein shows that some regions (1–5, 32–38, 60–67, 112–116, 157–162) are less well, or only poorly, defined; that is, they are mobile. The EF loop, around 85–90, repositions itself in response to changes in pH, and comparing the left-hand panel of Fig. 7.5a (pH 6) with the right-hand one (pH 8) shows this most convincingly. Figure 7.5b also shows the movement of the EF loop in a comparison of the high-pH crystallographic structure with the low-pH NMR structure.

Protein flexibility is better observed in solution by NMR methods. Using ^{1}H NMR, Molinari and co-workers have painstakingly derived a structure for the protein that in many features agrees with the crystal structures (Molinari *et al.*, 1996; Ragona *et al.*, 1997; Fogolari *et al.*, 1998). The NMR studies were carried out at pH 2, where the monomer predominates, simplifying the analysis. Similar results have also been obtained by other groups (Belloque and Smith, 1998; Forge *et al.*, 2000; Uhrinova *et al.*, 2000; Edwards *et al.*, 2002), the latter two using ^{13}C- and ^{15}N-labelled recombinant β-Lg to refine the complete structure in solution at pH 2. The core of the protein is essentially the same as that described by the crystallographic analyses (Molinari *et al.*, 1996; Ragona *et al.*, 1997; Fogolari *et al.*, 1998; Belloque and Smith, 1998; Kuwata *et al.*, 1998; Uhrinova *et al.*, 2000). However, the external loops and the position of the helix are modified relative to the crystal structures at neutral pH (Jameson *et al.*, 2002). Some ingenious protein engineering that introduced an additional disulphide bridge through Ala34Cys allowed NMR work to proceed at neutral pH, once again confirming the fold of the polypeptide chain (Sakurai and Goto, 2006).

No such monomer–dimer complication has dogged the NMR studies of the monomeric equine and porcine β-Lgs. Resonances for the native equine structure have been determined (Kobayashi *et al.*, 2000, 2002), but a final structure has not been published although comparisons with bovine

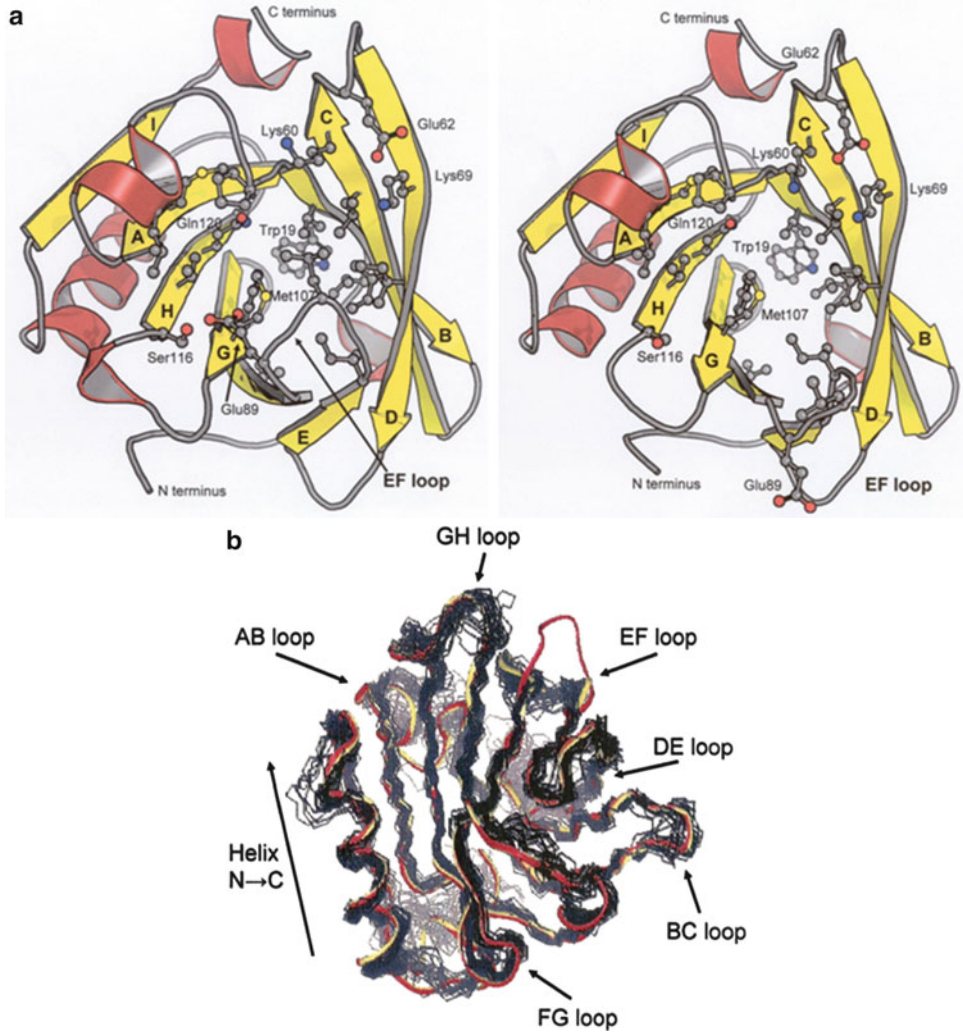

Fig. 7.5 (**a**) The structure of bovine β-lactoglobulin viewed into the central ligand-binding calyx at the bottom of which is Trp_{19}. In the *left image* of the structure at pH 6, the EF loop is in the closed position with the Glu_{89} buried and the side chain of Leu effectively occluding access to the calyx. Notice that the side chains within the calyx are hydrophobic with the exception of Gln_{120}. Met_{107} lies closer to the viewer than Phe_{105}, seen edge on, and these two side chains move to accommodate the ligand. The charged residues Lys_{60}, Glu_{62} and Lys_{69} are at the entrance and can interact with polar head groups of ligands. The *right-hand image* is the same view but of the structure at pH 8 where the EF loop has swung away providing access to the binding site. The drawing was made using PyMOL (2008). (**b**) Showing a superposition of 40 NMR structures at pH 2.5 with the high-pH (*red*) and low-pH (*yellow*) X-ray structures of bovine β-lactoglobulin. Most loops are clearly more mobile than the core sheet structure, and the pH-dependent movement of the EF loop is clearly visible (figure adapted from Uhrinova *et al.*, 2000, with permission. Copyright 2000, American Chemical Society)

β-Lg show a high degree of consistency. Studies on the porcine protein have also shown that it is monomeric at neutral pH (Ugolini *et al.*, 2001; Ragona *et al.*, 2003). Heterologous expression has also been reported such that a full NMR assignment is possible (Invernizzi *et al.*, 2004). What is revealed so far is that the NMR structure is consistent with that determined by X-ray crystallography. However, neither horse nor pig β-Lg NMR coordinate sets have yet been deposited.

7.7 Amino Acid Environments

Probing the environments of the various amino acid types was performed originally by protein chemistry methods which, though specific for amino acid type, could cause significant protein perturbation and therefore required careful interpretation (reviewed by Sawyer, 2003). The advent of site-directed mutagenesis has added considerably to studies of the stability, reactivity and environment of individual amino acid residues. Early work on the environment of specific amino acid types within bovine β-Lg was summarised by Townend *et al.* (1969) and found to be essentially correct when the X-ray structure emerged (Brownlow *et al.*, 1997; Bewley *et al.*, 1997; Qin *et al.*, 1998b). Although some studies have been carried out on the caprine and ovine proteins, which from the sequence identities are expected to be very similar to bovine, less was known about the monomeric β-Lgs, until the detailed NMR studies of the horse protein were performed (Fujiwara *et al.*, 1999; Kobayashi *et al.*, 2000) and the crystal structure and NMR details of the porcine protein published (Hoedemaeker *et al.*, 2002; Invernizzi *et al.*, 2004; D'Alfonso *et al.*, 2005). Some recent results on the reactivity of various amino acids are summarised below.

The free cysteine, Cys_{121}, in bovine β-Lg is an obvious target, and its pH-dependent availability and effect on dimer stability examined (Sakai *et al.*, 2000; Chamani, 2006). Cys_{121} is some way from the interface, but its reaction will interfere with the helix transmitting an effect that usually destabilises the dimer. Disulphide interchange occurs under denaturing conditions leading to aggregation (Creamer *et al.*, 2004), an effect that is affected by the genetic variant (Manderson *et al.*, 1998, 1999a, b). Mutation of Cys_{121} destabilises the structure somewhat but eliminates the disulphide interchange (Cho *et al.*, 1994b; Yagi *et al.*, 2003; Jayat *et al.*, 2004). An alternative cross-linking procedure using tyrosinase, and the modulator, caffeic acid, produces polymers with a molecular weight >300 kDa with the maximum rate occurring between pH 4 and 5 (Thalmann and Lötzbeyer, 2002). Another cross-linking approach has been to use a Ca^{2+}-independent microbial transglutaminase that does form polymers of β-Lg (Hemung *et al.*, 2009) but requires either heating or disulphide reduction of β-Lg (Sharma *et al.*, 2001; Eissa *et al.*, 2006). The transamination of β-Lg with low molecular weight amines identifies the residues in an amine-dependent manner. Thus, Gln 35, 59, 68 and 155 are transaminated with 6-aminohexanoic acid (Nieuwenhuizen *et al.*, 2004) and Gln 13, 68, 15/20 and 155/159 with 5-biotinamido-pentylamine (Hemung *et al.*, 2009).

The proximity of the potential quenchers cystine 66–160 to Trp_{61} and Arg_{124} to Trp_{19} means that the local environments are sensitive to small changes in structure and to their accessibility by quenchers (Busti *et al.*, 1998; Bao *et al.*, 2007; Harvey *et al.*, 2007; Edwards *et al.*, 2009). This in turn means that observed fluorescence changes, generated, for example, by ligand binding, must be interpreted with caution. Lysine methylation, acetylation or succinylation has produced material, far from native, that has antiviral activity (Chakraborty *et al.*, 2009; Sitohy *et al.*, 2010), and Caillard *et al.* (2011) have discussed the use of succinylated β-Lg as a suitable vehicle for oral drug delivery. Antiviral activity has also been shown for esterified β-Lg (Sitohy *et al.*, 2007) although at a level significantly lower than acyclovir.

Specific side-chain reactivity can also be explored by proteolysis, and the reader is referred to Volume 1B and Hernandez-Ledesma *et al.* (2008) for details of the bioactive peptides that have been produced from β-Lg by a variety of enzymes and procedures.

7.8 Solution Studies

Essentially every available technique has been applied to probe the physicochemical behaviour of β-Lg in vitro. Almost all of these studies have used the bovine protein and, unless specifically mentioned, it is this protein which is being described. The principal physicochemical parameters are given in Table 7.5.

Table 7.5 Selected molecular properties of bovine β-lactoglobulin

Number of amino acids	162	http://www.expasy.ch/cgi-bin/protparam
Total number of atoms (B variant)	2,596	http://www.expasy.ch/cgi-bin/proparam
Molecular formula (B variant)	$C_{817}H_{1316}N_{206}O_{248}S_9$	http://www.expasy.ch/cgi-bin/protparam
Monomeric M_r (B genetic variant) (Da)	18,281.2	http://www.expasy.ch/cgi-bin/protparam
M_r (B genetic variant) (Da)	18,278.8	Leonil *et al.* (1995)
Isoelectric point (B genetic variant, native) (pH)	5.407	Godovac-Zimmermann *et al.* (1996)
(Reduced and denaturing conditions)	4.968	Godovac-Zimmermann *et al.* (1996)
Theoretical isoelectric point (B variant)	4.83	http://www.expasy.ch/cgi-bin/protparam
Extinction coefficient: 1 mg/mL at 278 nm	0.961	Townend *et al.* (1960b)
Calculated extinction coefficient	0.919	http://www.expasy.ch/cgi-bin/protparam
Hydration (g H_2O/g protein)	0.46	Pessen *et al.* (1985)
Monomer hydrodynamic radius (nm)	2.04	Aymard *et al.* (1996)
Dimer hydrodynamic radius (nm)	3.19	Aymard *et al.* (1996)
Radius of gyration, dimer (nm)	2.1	Panick *et al.* (1999)
Radius of gyration, octamer (nm)	3.44	Timasheff and Townend (1964)
Sedimentation coefficient ($S°_{20w} \times 10^{13}$/s)	2.83	Cecil and Ogston (1949)
Monomer diffusion coefficient (10^{-11} m^2/s)	10.5	Aymard *et al.* (1996)
Dimer diffusion coefficient (10^{-11} m^2/s)	6.7	Aymard *et al.* (1996)
Intrinsic viscosity (mL/g)	3.4	Tanford (1961)
Partial specific volume (cm^3/g)	0.751	Svedberg and Pedersen (1940)
Axial ratio (dimer)	2:1	Green and Aschaffenburg (1959)
Dimer K_d (A genetic variant)		
pH 3.0, 10 mM NaCl, 293 K	3.07×10^{-3} M	Sakurai *et al.* (2001)
pH 6.5, 20 mM NaCl, 293 K	4.93×10^{-6} M	Sakurai *et al.* (2001)
pH 8.2, 130 mM NaCl, 293 K	1.96×10^{-5} M	Zimmerman *et al.* (1970)
Octamer dissociation constant pH 4.7, 274 K	4.55×10^{-12} M^3	Gottschalk *et al.* (2003)
Dipole moment (Debye)	698 (corrected for M_r)	Ferry and Oncley (1941)

7.8.1 Solubility

β-Lg, a globulin, is largely insoluble in distilled water and so can be precipitated and even crystallised by dialysis (Senti and Warner, 1948; Green *et al.*, 1956; Adams *et al.*, 2006). Salt increases the solubility quite dramatically: Polis *et al.* (1950) dissolved 1.8 g/L in water at the pI compared to 16.5 g/L at pH 5.2 in 0.2 M NaCl, a tenfold increase, and Treece *et al.* (1964) showed β-Lg B to be about five times more soluble than β-Lg A, a result that is perhaps counterintuitive on account of the A variant having an extra charge (Asp for Gly_{64}). Arakawa and Timasheff (1987) maintain that because the solubility is anomalous, much salt binding occurs because of the unique distribution of surface charge, and hence dipole, at neutral pH (Ferry and Oncley, 1941), a view shared by Piazza *et al.* (2002) and Bertonati *et al.* (2007). This conclusion has been given further weight in a systematic study of the solution properties (Holt *et al.*, 1999). Solubility curves for β-Lg around the pI (Grönwall, 1942) are often shown in undergraduate biochemistry texts (e.g. Voet and Voet, 2004). Salting out concentrated protein solutions, the other extreme, is the standard way of growing X-ray quality crystals of any β-Lg (Aschaffenburg *et al.*, 1965; Rocha *et al.*, 1996; Hoedemaeker *et al.*, 2002; Oksanen *et al.*, 2006), although dialysis against distilled water is an alternative (Adams *et al.*, 2006).

7.8.2 Molecular Size

Early studies of the M_r of bovine β-Lg under various conditions converged to a value of ~36,000 Da although at high dilution or low pH, a half-size component became significant. The association/dissociation data for bovine β-Lg are summarised in Table 7.5, and fuller discussions can be found elsewhere (Verheul *et al.*, 1999; Sakurai *et al.*, 2001; Gottschalk *et al.*, 2003; Invernizzi *et al.*, 2006; Bello *et al.*, 2008, 2011; Mercadente *et al.*, 2012). Ruminant β-Lg at neutral pH is mostly a dimer of two identical or near-identical subunits and at pH 2 at low ionic strength the monomer species predominates, with the core remaining pretty much in the native form, although both NMR and fluorescence studies show subtle changes (Mills and Creamer, 1975; Molinari *et al.*, 1996; Kuwata *et al.*, 1999; Uhrinova *et al.*, 2000). Low salt concentration at low β-Lg concentrations enhances dissociation (Aymard *et al.*, 1996; Renard *et al.*, 1998). For [β-Lg] < 0.3 mM, the K_d in 0.2 M NaCl of 1.00×10^{-5} M rises to 3.55×10^{-3} M in the absence of NaCl. Dissociation is also enhanced by thiol modification (Burova *et al.*, 1998) and increasing temperature (Aymard *et al.*, 1996; Bello *et al.*, 2008, 2011). These recent K_d values are in keeping with those derived elsewhere, which also show that the β-Lg genetic variants dissociate in the order A ≥ B > C (Timasheff and Townend, 1961; Thresher and Hill, 1997; Bello *et al.*, 2011). It has also been shown by Hill *et al.* (1996) that AA, BB and AB have K_d values of 1.5, 1.8 and 2.1×10^{-6} M in simulated milk ultrafiltrate buffer, pH 6.6 and 20°C. Recent work on the energetics of hydration involving both experimental and theoretical studies of dimer formation (Bello *et al.*, 2008) has shown that subunit association also involves 'burying' some 36 water molecules.

Between pH 3.5 and 6.5, with a maximum at pH 4.5, the bovine A variant forms octamers (i.e. four dimers), especially at low temperatures (Townend and Timasheff, 1960; Timasheff and Townend, 1961; Pessen *et al.*, 1985; Verheul *et al.*, 1999; Gottschalk *et al.*, 2003). Since the A variant has the external Gly64Asp mutation, the reason may be some form of carboxyl–carboxylate interaction (Sawyer and James, 1982). Indeed, Armstrong and McKenzie (1967) showed that carbodiimide modification of the carboxyls affects only the ability to octamerise. No crystals capable of full X-ray structure determination of the A variant at pH 4.6 have been obtained so that a direct view of the octamer remains elusive. Tetragonal crystal forms have been reported, from which it is tempting to speculate that they may reflect the likely 422 symmetry (Green and Aschaffenburg, 1959; Timasheff and Townend, 1964; Witz *et al.*, 1964). However, this need not be the case. Indeed, Mercadente *et al.* (2012) have shown very recently that in 0.1M NaCl and 45 microM protein, the dimer is the predominant form over the pH range 2.5 to 7.5 at 25ºC with no evidence of larger complexes. Interestingly, although neither determination was at pH 4.6, the salt-free bovine (Adams *et al.*, 2006) and the reindeer (Oksanen *et al.*, 2006) structures have four and eight subunits in the asymmetric unit, hinting at a larger structure than the dimer.

Over the pH range 8–9.5, slow time-dependent changes occur in β-Lg. At pH values above 8.5, reversible dissociation occurs (Georges and Guinand, 1960; Invernizzi *et al.*, 2006) and, above pH 9, the optical rotatory dispersion (ORD), circular dichroism (CD) and solubility change with time as the protein denatures irreversibly and aggregates (Groves *et al.*, 1951; Christensen, 1952; Herskovits *et al.*, 1964; Townend *et al.*, 1967). Addition of a thiol-blocking group can inhibit aggregation, implicating thiol oxidation and/or thiol/disulphide exchange in the formation of the heavier components (Roels *et al.*, 1966).

As regards the nonruminant protein, comparative pH studies by mass spectrometry on the pig and cow β-Lgs (Invernizzi *et al.*, 2006) find that the pig protein associates to a dimer distinct from that of the ruminants (Hoedemaeker *et al.*, 2002) at pH 4.0 but is monomeric at pH 6.0 and above. The K_d is 9 μM which is consistent with the 56 μM obtained by gel permeation at pH 3.0 (Ugolini *et al.*, 2001). Information on the equine protein indicates that it does not form dimers between pH 3.3 and 8.7 although incubation at

pH 1.5 for 2 h or treatment with 3 M urea at pH 8.7 was shown to produce some material with an apparent MW greater than the monomer (Ikeguchi *et al.*, 1997; Fujiwara *et al.*, 2001). A chaemeric form of the protein, retaining the cow core but with the horse loops, has been shown to form cow-like dimers (Ohtomo *et al.*, 2011).

7.9 Conformation and Folding

Early work using CD and the related technique of ORD together with ultracentrifugation and other techniques (reviewed by Sawyer, 2003) showed that bovine β-Lg underwent three pH-dependent conformational transitions between pH 2 and 10 which can be summarised as:

$$Q \leftrightarrow N \leftrightarrow R \rightarrow S$$

This pH-dependent conformational variation has been extended using ultrasonic, densimetric and spectroscopic studies (Taulier and Chalikian, 2001) which suggest that there are five distinct changes in conformation that lead to variations in protein hydration, compressibility and specific volume, the extra transitions over the above scheme being below pH 2 and above pH 10.

7.9.1 Q↔N

Bovine β-Lg variants A, B and C undergo the reversible Q↔N transition between pH 4 and 6 (Timasheff *et al.*, 1966b; McKenzie and Sawyer, 1967). The pH-dependent increase in sedimentation coefficient correlates with a contraction of the protein. The titration behaviour is consistent with a two-proton ionisation for β-Lg A while β-Lg B and β-Lg C follow a single proton transition. This is in keeping with the extra Asp_{64} in the A variant. In β-Lg C, one extra cationic residue per subunit, presumably His_{59}, is exposed upon increasing the pH. No aromatic residues are involved in this transition (Timasheff *et al.*, 1966a; Townend *et al.*, 1969) which is slightly surprising since His_{59}, Trp_{61} and Asp_{64} are located in the same region of the protein, albeit on the surface. No change is detected in the IR spectrum (Casal *et al.*, 1988). The changes have also been identified by NMR (Fogolari *et al.*, 1998, 2000; Kuwata *et al.*, 1999) and are generally fairly small. The core remains compact although the helix moves slightly relative to its position at neutral pH. Loops AB and CD adopt different positions, and loop EF is in the 'closed' position, preventing access to the central calyx. Glu_{89} is buried. Presumed movement of the helix caused by modification of the free Cys_{121} also leads to dissociation of the dimer (Zimmerman *et al.*, 1970; Iametti *et al.*, 1998; Burova *et al.*, 1998), but whether dissociation at low pH causes the movement or results from it is not yet clear.

7.9.2 N↔R

Between pH 6.5 and 7.8, the second reversible conformational change (N↔R), often called the Tanford transition, is observed (Groves *et al.*, 1951; Tanford *et al.*, 1959). In bovine β-Lg, this transition can be detected by a simple change in optical rotation ($[\alpha]_D$ is −25° at pH 6, but −48° at pH 8), by a decrease in the sedimentation coefficient (3.2–2.6 S) or possibly even a thermal denaturation peak (de Wit and Klarenbeek, 1981; Qi *et al.*, 1995, 1997). The change in sedimentation coefficient may result from protein expansion, shape variation (Tanford *et al.*, 1959; Timasheff *et al.*, 1966b) or increased dissociation (Georges *et al.*, 1962; McKenzie and Sawyer, 1967).

Upon increasing the pH, the buried carboxyl of the conserved Glu_{89} becomes exposed and ionised, with a positive enthalpy (Tanford and Taggart, 1961), arising from hydrogen bonding to the carbonyl of Ser_{116} (Brownlow *et al.*, 1997; Qin *et al.*, 1998a). The anomalous carboxyl ($pK_a = 7.3$), which titrates normally in urea- and alkali-denatured β-Lg (Tanford *et al.*, 1959), has also been observed in caprine β-Lg (Ghose *et al.*, 1968) and appears to be a conserved feature of β-Lgs since the residue is conserved in all species, except the fur seal in which it is Gln (Table 7.3; Ragona *et al.*, 2003; Cane *et al.*, 2005;

Edwards *et al.*, 2009). A Tyr has been shown to be involved (Townend *et al.*, 1969), and Tyr_{102} is in the vicinity of Glu_{89} although no significant movement is observed between N and R states (Qin *et al.*, 1998a). Cys_{121} also becomes more accessible to pCMB (Dunnill and Green, 1965) and $KAu(CN)_4$ (Sawyer and Green, 1979) when the pH is increased from 6 to 8, but it is some way from Glu_{89} and the EF loop. That the change affects access to the calyx has been shown by the careful binding studies of Ragona *et al.* (2003) monitoring the NMR signal from ^{13}C-labelled palmitic acid. The bound fraction is pH dependent with a half-site occupancy at about pH 5.5, two whole pH units below the H^+-mediated Tanford transition, but corresponding to the calculated pK_a of Glu_{89} in the 'closed' N state. The pH titration behaviour compared to the ligand-binding behaviour indicates an interesting shift in the pK_a of Glu_{89} presumably brought about by the sparingly soluble ligands binding to the small concentration of open form present at pH below 7. Recall that the calyx is apparently empty (Qvist *et al.*, 2008). No ligand binding is observed at pH 2 (Ragona *et al.*, 2000). Molecular dynamics simulation of the protonated and deprotonated Glu_{89} is consistent with the above behaviour (Eberini *et al.*, 2004)

Recently, Goto and his co-workers have provided a detailed explanation of the Tanford transition (Sakurai and Goto, 2006, 2007; Sakurai *et al.*, 2009). Using the Ala34Cys mutant that stabilises the β-Lg dimer, three distinct phases were identified by NMR on the nano- to millisecond timescale. First, chemical shift differences revealed that a group associated with the G strand has a pK_a of 6.9 which they assigned to Glu_{89}. Next, on the microsecond timescale, consideration of the relaxation data allowed significant fluctuations of the hydrogen bonds of Ile_{84}, Asn_{90} and Glu_{108} to be identified above pH 7.0. These residues are at the hinge of the EF loop. On still longer a timescale, differences in signal intensity associated with residues in the EF and GH loops together with some on the D strand correlate with the pH change. Together, these data were interpreted as initially Glu_{89} is deprotonated allowing fluctuation of the hydrogen bonding of residues Ile_{84}, Asn_{90} and Glu_{108} which in turn allows strand D and loops EF and GH to rearrange, thereby allowing access to the internal binding site. By varying the humidity of the crystals, Vijayalakshmi *et al.* (2008) have produced a form of β-Lg in which the EF loop in one subunit is closed but open in the other, from which it appears that the Tanford transition does not involve inter-subunit cooperativity.

Ragona *et al.* (2003) have shown that for porcine β-Lg, binding does indeed occur but only at pH values above 8.6. The pK_a of Glu_{89} was measured at 9.7, and the calculated value from the porcine structure is 7.4. In the light of this observation, it would be interesting to measure the pH dependency of ligand binding in other β-Lgs reported not to bind ligands (Pérez *et al.*, 1993).

7.9.3 R→S

The third, irreversible, conformational change is the alkali denaturation of β-Lg observed by many (Groves *et al.*, 1951; Townend *et al.*, 1960a; Timasheff *et al.*, 1966a; Hui Bon Hoa *et al.*, 1973; Purcell and Susi, 1984; Casal *et al.*, 1988; Mercadé-Prieto *et al.*, 2008).

7.9.4 Folding

The 'folding problem' in biology is one that has fascinated scientists for many years: given an amino acid sequence, can one predict its native 3D structure? (Richards, 1991) Because β-Lg is a relatively small β-barrel protein, it is a useful subject for such studies. Further, the existence of extra helical segments observed during the refolding makes the reason for the switch from helix to sheet all the more intriguing. It is worth noting that essentially all folding studies are performed by refolding the unfolded protein, usually by diluting the urea or guanidinium chloride used to achieve unfolding. Thus, it is important that suitable conditions are found which permit refolding to the native state. With β-Lg such conditions are readily attainable although a great deal of work over the years has centred round the irreversible denaturation that is important commercially.

Reversible unfolding–refolding of bovine β-Lg at ambient temperatures has been examined at low pH (Tanford and De, 1961; Pace and Tanford, 1968; Alexander and Pace, 1971; Hamada and Goto, 1997; Ragona *et al.*, 1999) and at neutral pH (McKenzie *et al.*, 1972; Hattori *et al.*, 1993; Creamer, 1995; Subramaniam *et al.*, 1996). The methods used to monitor the process include spectrophotometry, CD, NMR, fluorescence, DSC, ligand-binding and antibody recognition. The problem of disulphide interchange identified by McKenzie *et al.* (1972) was addressed further by Cupo and Pace (1983) who used a thiol-modification approach to show that the extra, external disulphide destabilised the structure. Sakai *et al.* (2000) showed that a modified Cys_{121} produced a molten globule-like structure at pH 7.5 while at pH 2.0 the protein remained native-like. Creamer (1995) minimised possible interchange by working rapidly at pH 6.7, and his study showed the stabilising effects of added ligand. To address the folding problem in more structural detail, SAXS, fluorescence, NMR, CD and proton exchange studies have all been used (Kuwajima *et al.*, 1987; Hattori *et al.*, 1993; Hamada *et al.*, 1995, 1996; Hamada and Goto, 1997; Arai *et al.*, 1998; Kuwata *et al.*, 1998, 2001; Mendieta *et al.*, 1999; Ragona *et al.*, 1999; Forge *et al.*, 2000). A refolding scheme has emerged whereby β-strands F, G and H and the main α-helix are formed within 2 ms with concomitantly, some non-native α-helix around the N-terminal part of the A strand. Thus, the helix and sheet E–H form first together with the C-terminal half of the A strand, before the N-terminal part of the A strand with strands B–D completes the folding. Sakurai *et al.* (2009) suggest that the non-native helix clearly identified in predictions is a means of preventing unwanted hydrogen bonding during folding. They summarise the folding of β-Lg in terms of an unfolded state passing through intermediate states with identifiable non-native helix, to produce, eventually, the N state. It is not clear that the molten globule state is on the folding pathway, since most reports of its existence involve variation in temperature or solution dielectric. Figure 7.6 summarises this proposed scheme. Unfolding

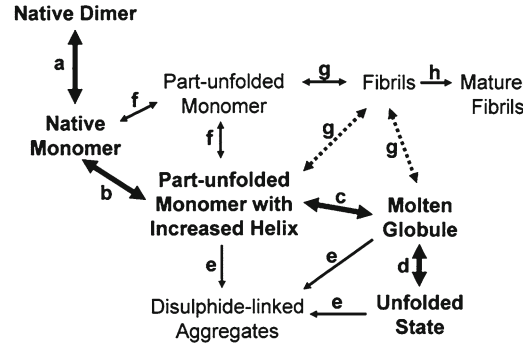

Fig. 7.6 A scheme showing the stages involved in the proposed folding/reversible unfolding (*bold*—**a**, **b**, **c**, **d**) and irreversible denaturation (**e**, **f**, **g**, **h**) of bovine β-lactoglobulin. It is probable that the dimer can undergo some unfolding/denaturation without first becoming a monomer, and certainly non-covalent interactions are present in the various aggregates and fibrils. The molten globule state can be isolated by changing the dielectric constant, but is most probably a transient state in the normal folding pathway

studies on caprine β-Lg have shown it to be slightly less stable than either bovine β-Lg A or B (Alexander and Pace, 1971).

Work with equine β-Lg, a monomer under physiological conditions, has identified both a reversible molten globule state and one in which there is also a greater-than-native content of α-helix at pH 1.5 (Ikeguchi *et al.*, 1997; Fujiwara *et al.*, 1999; Kobayashi *et al.*, 2000). Ikeguchi and colleagues have been able to equate the molten globule state with the burst phase state found with bovine β-Lg, and they observed protection from hydrogen exchange of residues on strands A, F, G and H, and on the helix. Their more recent studies also reveal the presence of non-native helix at alkaline pH as well as at low temperatures (Nakagawa *et al.*, 2006, 2007; Matsumura *et al.*, 2008). They too have relied both upon stabilisation of intermediates in water–alcohol solvents (e.g. Matsumura *et al.*, 2008) and site-directed mutagenesis (Yamada *et al.*, 2006; Nakagawa *et al.*, 2006, 2007) to study the folding.

Porcine β-Lg has been examined by Molinari and her co-workers who have also identified an intermediate state with non-native helix (D'Alfonso *et al.*, 2005; Ugolini *et al.*, 2001). They interpret the unfolding, as with the bovine

and equine proteins, as a process requiring intermediates, with the porcine protein being less stable at both pH 2 and pH 6 than bovine β-Lg.

7.10 Denaturation

Arguably the largest topic discussed in the bovine β-Lg literature concerns its denaturation on its own, but increasingly in the past couple of decades, in mixtures with other proteins, carbohydrates and lipids. This enormous literature reflects the commercial importance of the effects of the various processing techniques on whole milk, skim milk and whey protein preparations in which β-Lg is seen to play a crucial role. Indeed, as recently pointed out by de Wit (2009), a significant amount of the recent literature merely repeats studies done before much of the electronic archive, which often begins only in mid-1990s. However, cursory literature surveying is not the only reason for revisiting the measurements, since monitoring techniques have become significantly more sensitive which has highlighted the fact that the behaviour of β-Lg is sensitive to small changes in the conditions, especially at the protein concentrations that occur in milk (Qi *et al.*, 1995, 1997; Holt *et al.*, 1998, 1999). In this section, 'denaturation' is taken to mean the generation, often irreversibly, of insoluble material.

Briefly, bovine β-Lg appears to denature through an initial dissociation from dimer to monomer followed by a change in the polypeptide chain conformation and subsequent aggregation. Because of the free thiol in β-Lg, disulphide interchange can also occur leading to oligo-/polymer formation, involving the 66–160 cystine which is generally the more accessible, although intra-subunit exchange also can occur (McKenzie and Shaw, 1972; Manderson *et al.*, 1999b; Creamer *et al.*, 2004). Depending upon the precise conditions used, particulate or fibrous material can be formed and recently, amyloid fibrils have been made to form most efficiently through low pH heating (Foegeding, 2006; Hamada *et al.*, 2009). The denaturant can be alkali (Mercadé-Prieto *et al.*, 2008), heat (e.g. Qi *et al.*, 1997; Carrotta *et al.*, 2003; Creamer *et al.*, 2004; Bhattacharjee *et al.*, 2005; Tolkach and Kulozik, 2007), cold (Katou *et al.*, 2001; Davidovic *et al.*, 2009), pressure (e.g. Iametti *et al.*, 1997; Belloque *et al.*, 2000; Considine *et al.*, 2007), organic compounds (e.g. D'Alfonso *et al.*, 2002; Dar *et al.*, 2007), metal ions (e.g. Stirpe *et al.*, 2008; Gulzar *et al.*, 2009) or metal surfaces (Changani *et al.*, 1997; Jun and Puri, 2005; Bansal and Chen, 2006). Studies with combinations of these are also common (e.g. Aouzelleg *et al.*, 2004). Even various forms of radiation can be used. For example, Bohr and Bohr (2000) have examined the effects of microwave radiation by SAXS suggesting that the effects are nonthermal which is in keeping with the observation that microwave treatment at a non-denaturing temperature enhances susceptibility to proteolysis (Izquierdo *et al.*, 2007). Similarly, the aggregation by γ-radiation followed by SAXS is significant in solution, though not in the solid state, and increases with decreasing protein concentration (Oliveira *et al.*, 2006). It appears that the cross-linking initiated by $OH^{\bullet}$ radicals is through tyrosine side chains, most of which are solvent accessible (Townend *et al.*, 1969; Brownlow *et al.*, 1997).

It is not yet clear in molecular detail how each of these denaturing agents acts to yield insoluble aggregates, although it is probable that the mechanism is agent-dependent and that several of the stages may be common (Fox, 1995; Qi *et al.*, 1997; Manderson *et al.*, 1998, 1999a, b; Edwards *et al.*, 2009; see also Volume 1B). The effects are also modulated by the presence of ligands (e.g. Boye *et al.*, 2004; Considine *et al.*, 2005; Busti *et al.*, 2005), generally thought to stabilise the folded protein. What has become clear is that the mechanism depends on the pH, the ionic strength and the nature of the ions, the concentration *and purity* of the protein, the dielectric constant and the temperature (Dufour and Haertlé, 1993; Li *et al.*, 1994; Relkin, 1996; Renard *et al.*, 1998; Foegeding, 2006; Krebs *et al.*, 2007). Distinguishable effects also derive from the genetic variant (Hill *et al.*, 1996; Manderson *et al.*, 1998, 1999a, b; Holt *et al.*, 1998). An attempt to illustrate the various stages

that have been identified both in the unfolding–refolding and the denaturation processes that can lead to β-Lg coming out of solution is shown in Fig. 7.6. The conformational changes discussed above are also implied since the denaturation occurs more readily, the more open is the structure.

The situation is somewhat different with the monomeric equine and porcine proteins that lack a free thiol. Indeed, there is little published on the denaturation of the nonruminant β-Lgs, although there is on their unfolding and refolding. The equine and porcine proteins are not stable in acid solution, unlike the bovine protein (Ikeguchi *et al.*, 1997; Ugolini *et al.*, 2001; Burova *et al.*, 2002; Invernizzi *et al.*, 2006; Ohtomo *et al.*, 2011). Yamada *et al.* (2005) have compared the heat-denatured state of equine β-Lg with that obtained in acid, finding them to be similar but distinct from the cold-denatured state which, by CD, SAXS and ultracentrifugation, appeared to be expanded and chain-like with more α-helix than the more compact acid-denatured protein, not unlike the intermediates of the bovine protein.

As one of the consequences of protein denaturation is the formation of precipitate, in addition to food scientists, the process has recently attracted the attention of the nanotechnology (Krebs *et al.*, 2009; Hirano *et al.*, 2009) and soft matter physics (Donald, 2008) communities.

7.11 Binding Studies

The first report of a ligand bound to β-Lg appears to be that of oleic acid by Davis and Dubos (1947), followed a few years later by Groves *et al.* (1951) who observed that 2 mol/mol (36 kDa) SDS were bound to the protein in a manner that stabilised it against thermal denaturation. It was not until the 1960s, however, that binding constants began to be determined (Wishnia and Pinder, 1966) by equilibrium dialysis, and then Futterman and Heller (1972) using fluorescence happened upon the retinol-binding ability, unaware of the family relationship between retinol-binding protein (RBP) and β-Lg which only emerged a decade later.

The number of individual ligands reported to bind to β-Lg is now probably well in excess of 200, many of the recent reports arising because of the increased interest in the use of the protein as a means of trapping labile molecules (e.g. Loveday and Singh, 2008) and volatile flavours and aromas in food (Kühn *et al.*, 2006; Guichard, 2006). Table 7.5 of Sawyer (2003) contained the majority of ligands identified before 2000 by methods such as equilibrium dialysis, fluorescence measurements both intrinsic and extrinsic, gel permeation/affinity chromatography, NMR and ESR. The past decade has seen those 111 entries for 76 distinct ligands increase rapidly, and so no such exhaustive table is included here; rather Table 7.6 provides a snapshot, giving examples of the methods employed, the diversity of the ligands and the variation in binding constants from the supra-millimolar to the sub-micromolar. Another significant table of ligand-binding information can be found in Tromelin and Guichard (2006).

Free fatty acids bind to bovine β-Lg in a manner dictated by the chain length, with the tightest binding being for palmitate (Spector and Fletcher, 1970). Direct X-ray crystallographic evidence for ligand binding came relatively recently if one neglects the heavy atom compounds used for phase determination (Green *et al.*, 1979) and the homology model of Papiz *et al.* (1986), bromododecanoic acid (Qin *et al.*, 1998b), palmitic acid (Wu *et al.*, 1999) and retinol (Sawyer and Kontopidis, 2000) which showed that they bind within the central calyx between the two β-sheets. Examination of the cavity shows that there is little room for a ligand longer than palmitate. Careful NMR studies with palmitate by Ragona *et al.* (2000, 2003) and Konuma *et al.* (2007) confirmed this internal binding site for palmitate. Loch *et al.* (2011) have recently provided both crystallographic and solution data that show C_8 and C_{10} fatty acids bind to the calyx. Figure 7.7a shows palmitate (C_{16}) bound in the calyx from which it can be seen that there is a degree of flexibility in two of the hydrophobic residues lining the pocket, Phe_{105} and Met_{107}. This flexibility is mirrored in the binding of the carboxylate head groups shown in Fig. 7.7b which superimposes the binding of

Table 7.6 Ligand-binding parameters for bovine β-lactoglobulin

Ligand	Protein[a] source	pH	n[b]	K_a (M^{-1})[c] AA	BB	AB	X	Method[d]	References
Fatty acids									
Caprylic acid (C_8)	Sigma	7.5	1		1.1		10^4	F	Loch *et al.* (2011)
Capric (C_{10})	Sigma		1		6.0		10^3	F	Loch *et al.* (2011)
Lauric acid (C_{12})	Pentex	7.4	1	0.5			10^5	ED	Spector and Fletcher (1970)
Myristic acid (C_{14})	In-house	7.0	0.33	3.03	3.03		10^6	F	Frapin *et al.* (1993)
Palmitic acid (C_{16})	Pentex	7.4	1	6.8			10^5	ED	Spector and Fletcher (1970)
Palmitic acid	In-house	7.0	0.93		1.00		10^7	F	Frapin *et al.* (1993)
Palmitic acid	Sigma/ In-house	7.0	1.03	2.28			10^5	UF	Wang *et al.* (1998)
Stearic acid (C_{18})	Pentex	7.4	1	1.7			10^5	ED	Spector and Fletcher (1970)
5-Doxylstearic acid	Sigma	7.0	1			1.25	10^6	ESR	Narayan and Berliner (1997)
SDS	In-house	7.5	1.5		3.1		10^5	ED	Ray and Chatterjee (1967)
SDS	In-house	7.0	0.92		4.35		10^6	F	Lamiot *et al.* (1994)
Oleic acid (C_{18}:1)	Pentex	7.4	1	0.4			10^5	ED	Spector and Fletcher (1970)
Linoleic acid (C_{18}:2)	In-house	7.0	0.83		5.26		10^6	F	Frapin *et al.* (1993)
Retinoids									
Retinoic acid	Sigma	7.0	0.92	4.8			10^5	F	Chu *et al.* (1996)
Retinoic acid	Sigma/ In-house	7.0	0.90	5.88			10^7	F	Wang *et al.* (1997)
Retinoic acid	Sigma	7.0	1.14	5.10	5.71		10^6	F	MacLeod *et al.* (1996)
Retinoic acid	Recombinant	7.0	0.81	2.56			10^7	F	Cho *et al.* (1994a)
Retinol	Sigma	7.0	1		5.0		10^7	F	Fugate and Song (1980)
Retinol	In-house	7.0	0.83		2.27		10^7	F	Dufour and Haertlé (1991)
Retinol	Recombinant	7.1	1	5.88			10^7	F	Katakura *et al.* (1994)
Retinol	W19Y mutant	7.1	1	4.17			10^7	F	Katakura *et al.* (1994)
Retinol	Recombinant	8.0	0.85	2.13			10^7	F	Cho *et al.* (1994a)
Retinol	Recombinant	8.0	1	2.63			10^6	ED	Cho *et al.* (1994a)
Retinol	In-house	3.0	0.90		1.18		10^7	F	Dufour *et al.* (1993)
Retinol	In-house	7.2	1			1.5	10^4	GPC	Puyol *et al.* (1991)
Retinol	Sigma	7.5	0.85			8.3	10^7	F	Laligant *et al.* (1995)
Immobilised *trans*-retinal	In-house	7.5	1	2.86			10^7	AC	Jang and Swaisgood (1990)
β-Ionone	In-house	3.0	1.08		1.67		10^6	F	Dufour and Haertlé (1991)
β-Ionone	Besnier	3.0	1			5.2	10^2	AC	Jouenne and Crouzet (2000)
Flavours									
2-Nonanone	Sigma	6.7	1		2.44		10^3	ED	O'Neill and Kinsella (1987)
2-Nonanone	Besnier	3.0	1			3.6	10^3	AC	Sostmann and Guichard (1998)
2-Nonanone	Besnier	3.0	1			1.25	10^3	SHA	Sostmann and Guichard (1998)
Butyl pentanoate	Besnier	3.0	1			5.34	10^2	AC	Pelletier *et al.* (1998)
Ethyl benzoate	Besnier, AB	3.0	1			6.77	10^2	C	Pelletier *et al.* (1998)
Ethyl heptanoate	Besnier, AB	3.0	1			1.43	10^3	AC	Pelletier *et al.* (1998)
Hexyl acetate	Besnier	3.0	1			5.69	10^2	AC	Pelletier *et al.* (1998)

(continued)

Table 7.6 (continued)

Ligand	Protein[a] source	pH	n[b]	K_a (M^{-1})[c] AA	BB	AB	X	Method[d]	References
Hexyl propionate	Besnier	3.0	1			1.13	10^3	AC	Pelletier *et al.* (1998)
*Iso*pentyl acetate	Besnier	3.0	1			1.52	10^2	AC	Pelletier *et al.* (1998)
Methyl heptanoate	Besnier	3.0	1			6.76	10^2	AC	Pelletier *et al.* (1998)
Propyl hexanoate	Besnier	3.0	1			1.23	10^3	AC	Pelletier *et al.* (1998)
Limonene	Besnier	5.0	1			3.15	10^3	AC	Jouenne and Crouzet (2000)
Vanillin	Besnier	6.65	14			8.0	10^4	UF	Relkin and Vermersh (2001)
Vanillin	Besnier	3.0	1			3.19	10^2	AC	Reiners *et al.* (2000)
γ-Octalactone	Besnier	3.0	1			4.5	10^2	AC	Tromelin and Guichard (2006)
γ-Octalactone	Sigma	7.0	3			0.77	10^2	UF	Guth and Fritzler (2004)
Biological molecules									
Cholesterol	Sigma/ in-house	7.0	1.18	2.87			10^7	F	Wang *et al.* (1997)
CO-haem	In-house	7.0	1		2.0		10^6	S	Marden *et al.* (1994)
Ergosterol	Sigma-in-house	7.0	1.01	1.60			10^8	F	Wang *et al.* (1997)
Haemin	In-house, B	7.0	0.85		4.0		10^6	F	Dufour *et al.* (1990)
Protoporphyrin IX	In-house	7.0	0.75		2.5		10^6	F	Dufour *et al.* (1990)
NBD-didecanoyl-phosphatidyl-ethanolamine	Sigma	7.4	1			1.2	10^6	F	Martins *et al.* (2008)
Peptide β-Lg 142–148	Sigma	6.8	3	2.0			10^3	ITC	Roufik *et al.* (2006)
Vitamin D_2	Sigma	7.0	1.00	2.04			10^8	F	Wang *et al.* (1997)
Vitamin D_3	Sigma/home	7.0	1.01	2.78			10^7	F	Wang *et al.* (1997)
Sucrose oleate	Sigma	7.0	1			3.77	10^4	F/ED	Clark *et al.* (1992)
Sucrose stearate	Sigma	7.0	1			4.35	10^5	F/ED	Clark *et al.* (1992)
Hydrocarbons									
Butane	In-house	2.06	1	1.85	2.22		10^3	ED	Wishnia and Pinder (1966)
Heptane	Nut. Bio. Corp.	6.8	0.35			4.8	10^5	GLC	Mohammadzadeh *et al.* (1969)
Benzenoid molecules									
Toluene	In-house	5.8	1	4.5			10^2	ED	Robillard and Wishnia (1972)
Sodium polystyrene sulphonate	Sigma	6.27	16.8			1.91	10^4	CE	Hallberg and Dubin (1998)
Bromophenol blue	Miles B	9.35	1		1.57		10^4	S	Waissbluth and Grieger (1973)
p-Nitrophenol phosphate	In-house	6.0	1	3.2	1.6	1.4	10^4	F	Farrell *et al.* (1987)
Pyridinium bromide	In-house	7.5	1.5		2.7		10^4	ED	Ray and Chatterjee (1967)
1-Anilino-8-naph-thalene sulphonate	Sigma	2.3	2		1.40		10^4	F	D'Alfonso *et al.* (1999)
1-Anilino-8-naph-thalene sulphonate	Sigma	8.2	2		1.10		10^3	F	D'Alfonso *et al.* (1999)
Methyl orange	In-house	7.5	1	1.0	1.0		10^4	ED	Ray and Chatterjee (1967)

(continued)

Table 7.6 (continued)

Ligand	Protein[a] source	pH	n^b	K_a (M^{-1})[c] AA	BB	AB	X	Method[d]	References
Other									
Calcium ion	Sigma	7.4	3			1.3	10^2	ISE	Jeyarajah and Allen (1994)

[a]Genetic variant is given when specified. AB denotes mixed A and B variants which, when unspecified is assumed
[b]Number of binding sites monomer. Where this value is given as 1, no specific determination is reported
[c]The association constants are shown for genetic variants AA, BB or AB multiplied by the value in the column headed X
[d]Methods based upon *GLC* gas liquid chromatography; *F* fluorescence; *ED* equilibrium dialysis; *ESR* electron spin resonance; *AC* affinity chromatography to immobilised ligand; *ISE* ion-sensitive electrode; *ITC* isothermal titration calorimetry; *CE* capillary electrophoresis; *S* spectrophotometry; *C* chromatography; *GPC* gel permeation chromatography; *UF* ultrafiltration; *SHA* static headspace analysis; *Nut. Bio. Corp.* Nutritional Biochemical Corporation

C_8 to C_{18} fatty acids. Structures for the saturated fatty acids C_{12}, C_{14}, C_{16}, C_{18} have recently been published by Loch *et al*. (2012).

The pH dependence shows that binding increases with increasing pH (Spector and Fletcher, 1970; Frapin *et al.*, 1993) in accord with the movement of the EF loop that acts as a lid to the cavity (Qin *et al.*, 1998a, b; Wu *et al.*, 1999; Ragona *et al.*, 2000, 2003), the NMR studies showing that essentially no palmitate was bound at and below pH 3, consistent with the earlier report using fluorescence changes (Frapin *et al.*, 1993). The presence of Lys_{60} and Lys_{69} at the mouth of the cavity allows interaction with the acidic group of acid ligands, but alcohols like dodecanol and of course retinol can also bind tightly (Futterman and Heller, 1972; Hemley *et al.*, 1979; Lamiot *et al.*, 1994). Conversely, the positively charged *N*,*N*,*N*-trimethyl-dodecylammonium ion appears not to bind but to precipitate β-Lg (Waninge *et al.*, 1998; Lu *et al.*, 2006), or at least to bind differently (Magdassi *et al.*, 1996) in keeping with the presence of the positively charged sentinel lysines.

Before the publication of the crystal structure of the retinol-β-Lg complex (Kontopidis *et al.*, 2002), it was unclear where retinol bound to the molecule. Dufour *et al.* (1994) and Narayan and Berliner (1997, 1998) using fluorescence and fluorescence resonance energy transfer (FRET) measurements reported that retinol and fatty acid could bind simultaneously. Another FRET study placed the retinol/ANS binding site closer to Trp_{61} than to Trp_{19} (Lange *et al.*, 1998). In contrast, Puyol *et al.* (1991) used equilibrium dialysis to show that retinol and palmitate could compete for binding to β-Lg, and similarly, Kontopidis *et al.* (2002) showed that only palmitate could be detected in the calyx when β-Lg was co-crystallised from a ligand mixture; there was no indication of a second site. This same study showed directly retinol binding in the calyx (Fig. 7.7a).

While there is little difficulty accommodating ring compounds like toluene within the calyx of β-Lg, it is less clear that this is the binding site for larger, fused-ring compounds. Robillard and Wishnia (1972) showed there were two binding sites, one tight, the other weaker, which could conceivably both be within the cavity. However, as binding abolished octamer formation in the A

Fig. 7.7 (continued) positively charged Lys_{60} or Lys_{69}. The two residues that reposition their side chains on ligand binding, Phe_{105} and Met_{107}, are shown as *grey sticks*. The drawing was made using PyMOL (2008), and those fatty acids without an associated PDB code are unpublished results from the author's laboratory. (**c**) Another view of β-lactoglobulin rotated approximately 90° anticlockwise from that shown in Fig. 7.5a, showing the external binding site of vitamin D_3 (PDB code: 2gj5) at the C-terminal end of the helix and involving residues between 137 and 148. This site is that in which HgI_3^- binds (Papiz *et al.*, 1986). A third binding site identified from NMR shifts by Lübke *et al.* (2002) as that at which β-ionone appears to bind is at the other end of the helix around Tyr_{102}, Leu_{104} and Asp_{129}. The drawing was made using PyMOL (2008)

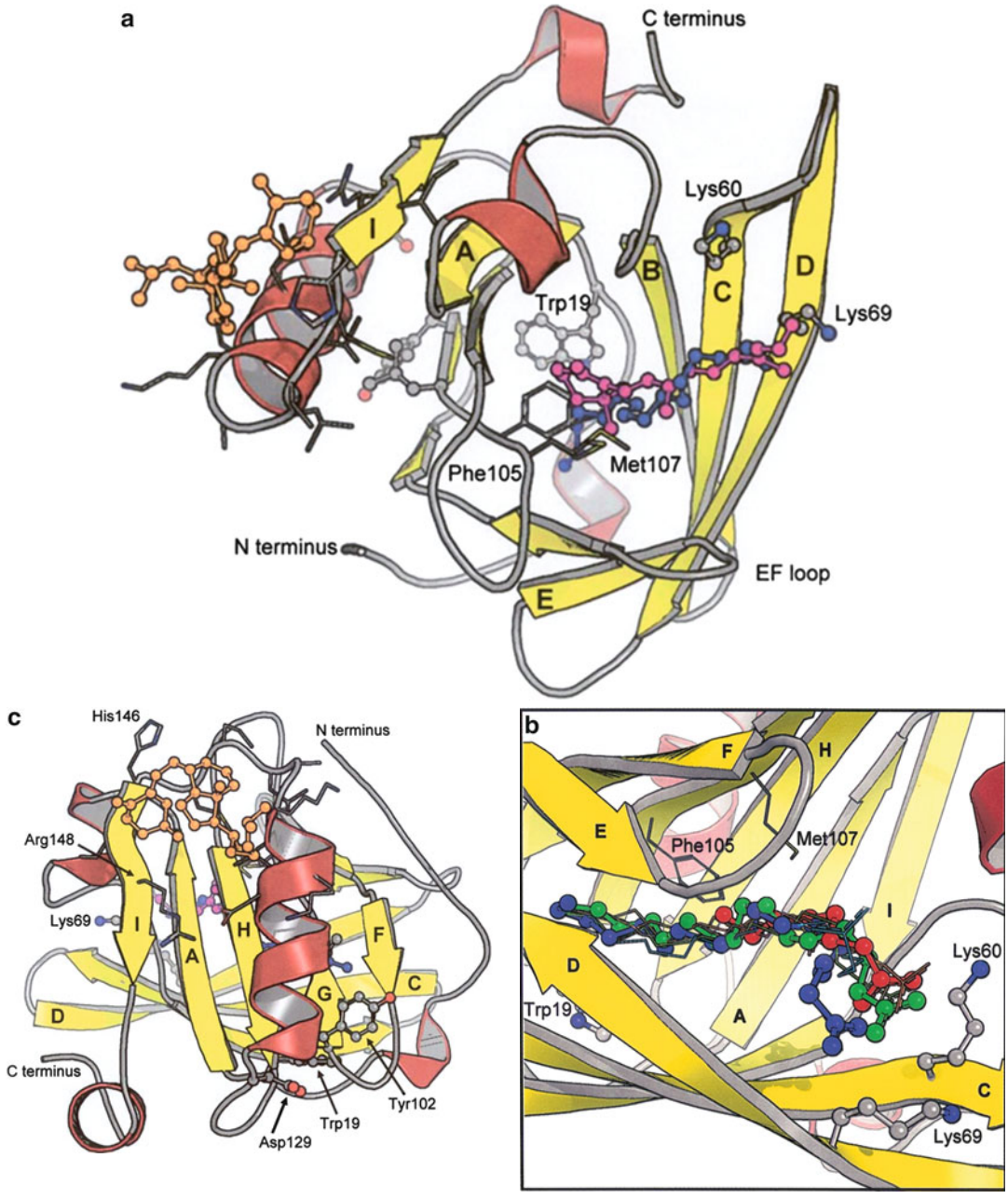

Fig. 7.7 (**a**) A view similar to that of Fig. 7.5a showing the superposition of palmitate (*blue*) and retinol (*magenta*) in the central calyx of β-lactoglobulin. The EF loop is in the open position, and the two side chains, Phe_{105} and Met_{107}, that undergo significant repositioning on binding are shown as *sticks*. The position of the external binding site of vitamin D_3 (*orange*; PDB code: 2gj5) is shown on the *left* near the helix. The drawing was made using PyMOL (2008). (**b**) A close-up view of the ligand-binding calyx showing the superposition of fatty acids whose coordinates have been determined by X-ray techniques. Caprylate (C_8, *red*, 3nq9), palmitate (C_{16}, *green*, 1gxa) and stearate (C_{18}, *blue*) are shown as ball-and-stick, whereas caproate (C_{10}, *salmon*, 3nq3), Br-laurate (C_{12}, *orange*, 1bso), myristate (C_{14}, *bright orange*), pentadecanoate (C_{15}, *split pea*) and margarate (C_{17}, *cyan*) are shown as *sticks*. Note the methyl end of the longest stearate molecule is bent back towards the opening. The considerable flexibility at the carboxylate end is evident and, interestingly, few direct interactions appear to be made with the

genetic variant, the second site was probably elsewhere. Lovrien and Anderson (1969) found two somewhat different anionic binding sites for *N*-methyl-2-anilino-6-naphthalenesulphonate at pH 8 but only one at pH 6, probably that for L-anilino-8-naphthalenesulphonate (ANS) (Mills and Creamer, 1975). D'Alfonso *et al.* (1999) found significant pH and ionic strength dependence for ANS with two distinct types of behaviour, concluding that the interaction was largely electrostatic, and an electrostatic analysis of the protein structure indicated that there may be more than one binding site for negatively charged ligands (Collini *et al.*, 2000, 2003; Fogolari *et al.*, 2000; Considine *et al.*, 2005). Kontopidis *et al.* (2004) found that vitamin D_2 and cholesterol bound independently in the calyx site so that the principal binding site is in the central calyx which is capable of accommodating quite sizeable molecules. Zsila and his colleagues have shown *cis*-parinaric acid (C_{18}:Δ_4) and piperine bind close to, or in, the calyx (Zsila *et al.*, 2002; Imre *et al.*, 2003), and also bilirubin (Zsila, 2003), protoporphyrin IX (Tian *et al.*, 2006) and norfloxacin (Eberini *et al.*, 2006), which are far from linear, also bind. These and other binding studies have led to an assessment by Konuma *et al.* (2007) that ligand binding to the β-Lg calyx is 'promiscuous'.

Following the work of Wang *et al.* (1997), who found 2 mol of vitamin D_2/mol of β-Lg, a paper by Yang *et al.* (2008) provides direct evidence of an external binding site for vitamin D_3, which is at the end of the helix and involves residues around 137–148 (Fig. 7.7c). This is not the binding site for β-ionone discovered by Guichard and colleagues using NMR (Lübke *et al.*, 2002; Tromelin and Guichard, 2006) which is close to Tyr_{102}, Leu_{104}, Asp_{129} and Gln_{120} on the outer surface of the protein not far from Trp_{19}. Neither is it any of those suggested by Eberini *et al.* (2006) from modelling and experimental ligand-binding studies, although it does appear as one of the eight sites found by the programs GRAMM and AUTODOCK (Guth and Fritzler, 2004). It is, however, the site of one of the heavy metal derivatives, HgI_3^-, used in the original crystal structure analyses (Green *et al.*, 1979; Papiz *et al.*, 1986) and is also that inferred from the work of Busti *et al.* (2005) and observed for ANS (G. B. Jameson, personal communication). Tegoni *et al.* (1996) have identified the same inter-subunit site in another lipocalin, odorant-binding protein. Thus, there is now direct evidence for two distinct binding sites and fairly clear evidence for a third, independent site, but the existence of others cannot be ruled out.

There is significant commercial interest in the binding of flavours and aromas to milk proteins (O'Neill and Kinsella, 1988; Kühn *et al.*, 2006), and consequently there are a large number of specific studies on binding to β-Lg. The variety of methods of analysis used has led to some discrepancies in the results. However, if measurements are made under the same conditions, then it is possible to compare ligands in a fairly meaningful way. Some progress towards rationalising ligand shape has been made using QSAR methods (Guth and Fritzler, 2004; Tromelin and Guichard, 2006). The conclusions reached disagree in detail but are self-consistent. What is more, Guichard and her colleagues have been able to group 85 ligands into three classes and to find distinctive characteristics for each that map to the structure of β-Lg (see Guichard, 2006).

Finally, one intriguing possibility, that of tailoring the binding site to suit a particular, unnatural ligand (McAlpine and Sawyer, 1990; de Wolf and Brett, 2000; Skerra, 2008), has become a realistic goal with the modification of the calyx of bilin-binding protein from *Pieris brassicae* to accept fluorescein with a nanomolar binding constant (Beste *et al.*, 1999; Vopel *et al.*, 2005).

7.11.1 Macromolecule Binding

It is not surprising that a protein with a wide variety of possible ligands also interacts with other proteins. There are numerous studies of the interactions between milk proteins, many resulting from the milk processing and food industries, which are out of the scope of this chapter. If heated, bovine β-Lg interacts with α-lactalbumin (Hunziker and Tarassuk, 1965) in a way that modifies the denaturation of α-lactalbumin

(Gezimati *et al.*, 1997). β-Lg also interacts with several of the caseins, perhaps the best characterised of which is with κ-casein (Sawyer, 1969; Hill, 1989; Lowe *et al.*, 2004), where the principal interaction is a disulphide linkage. Cytochrome *c* interacts with β-Lg ($K_d = 20$ μM) such that the Cys_{121} of β-Lg can reduce the haem iron (Brown and Farrell, 1978) at a rate that depends upon the genetic variant and which implies some rearrangement of β-Lg or the use of a mediator.

Specific receptor binding in calf intestine has been observed by Papiz *et al.* (1986), and specific receptors have also been reported in bovine germ cells and in a murine hybridoma (Mansouri *et al.*, 1997; Palupi *et al.*, 2000). Uptake by Caco-2 cells is reported by Puyol *et al.* (1995), Riihimaki *et al.* (2008) and by Jiang and Liu (2010), who show that β-Lg can successfully deliver linoleic acid, which may have anticancer implications. Further, it has been shown that the human lipocalin-interacting membrane receptor, expressed in the intestine, can recognise and internalise β-Lg (Fluckinger *et al.*, 2008). The binding of modified β-Lg to CD4 receptors has been noted (Neurath *et al.*, 1996). β-Lg has been found to inhibit the adhesion of bacteria that express the particular S-fimbrae to ileostomy glycoproteins by binding to the glycoprotein with dissociation constants as small as 13.5 nM (Ouwehand *et al.*, 1997). Moderate heat has little effect but reduction effectively abolishes this binding. Proteolysed fragments, rather than whole β-Lg, also have biological activity (Pellegrini, 2003), but recently Chaneton *et al.* (2011) have shown that the whole protein also has antibacterial activity against Gram-positive bacteria.

Immunological cross-reactivity has long been used as a convenient means of identifying the presence of β-Lg (Phillips *et al.*, 1968; Restani *et al.*, 1999; Suutari *et al.*, 2006), although it has not always provided unambiguous results. Thus, the cross-reaction between anti-bovine β-Lg and human milk results from the presence of dietary bovine β-Lg (Axelsson *et al.*, 1986; Fukushima *et al.*, 1997) in keeping with reports that β-Lg can cross membranes like the placenta (Szepfalusi *et al.*, 2000; Edelbauer *et al.*, 2004). The allergic behaviour of milk proteins has been studied extensively (see reviews by Crittenden and Bennett, 2005; Monaci *et al.*, 2006) from which it transpires that one of the significant allergens is β-Lg, also called Bos d 5 allergen (Lebenthal *et al.*, 1970).

A review of the identified epitopes on β-Lg to IgG, IgA and IgE antibodies and T- and B-cell determinants shows that they cover much of the surface, including the flexible loops (Clement *et al.*, 2002), but the reactivity with anti-β-Lg IgE of synthetic peptides matching these epitopes varies considerably, in reasonable agreement with tryptic, chymotryptic and peptic digests of β-Lg and other peptide scanning studies (Kurisaki *et al.*, 1982, 1985; Williams *et al.*, 1998; Jarvinen *et al.*, 2001). Niemi *et al.* (2007) have confirmed one of the minor epitopes (CC′) from the study by Clement *et al.* (2002) in a crystal structure analysis of the Fab fragment of an IgE, binding to the discontinuous regions of β-Lg 18–22, 43–47, 55–59, 65–70, 126–128, 153–162 and burying an area of some 890 $Å^2$, the key interaction being between a small cavity close to Trp_{19}/Glu_{44} and Arg_{101} on the antibody. Interestingly, this is not far from a site identified by Lübke *et al.* (2002).

Antibodies can also be used to probe the correct and complete (re-)folding of β-Lg (Kaminogawa *et al.*, 1989; Takahashi *et al.*, 1990; Hattori *et al.*, 1993). Hattori *et al.* (1993) found that β-Lg on refolding in vitro did not regain a conformation that was recognised by some of their monoclonal antibodies raised to the region containing Trp_{19}. This local structural variation was also found by Subramaniam *et al.* (1996) using Trp phosphorescence. Chatel *et al.* (1996) could not distinguish by using polyclonal, or four monoclonal, antibodies between native β-Lg and recombinant protein produced in *E. coli* and solubilised in urea before purification, although Katakura *et al.* (1997) were able to detect a slight difference from recombinant β-Lg produced in yeast. The monoclonal antibodies used in these two studies were different.

Reversing this approach and trying to remove the antibody binding to reduce the potential allergenicity is important to the food industry, and thermal processing (Davis and Williams, 1998; Mierzejewska and Kubicka, 2006), pressure (Chicon *et al.*, 2009), hydrolysis (Gestin *et al.*, 1997; Moreno, 2007), modification (Buetler *et al.*, 2008), conjugation to carbohydrate (Hattori *et al.*, 2004; Aoki *et al.*, 2006; Taheri-Kafrani *et al.*, 2009), glycosylation through protein engineering (Tatsumi *et al.*, 2012) and even γ-radiation (Kaddouri *et al.*, 2008) have all been examined, as has selective allergen removal (Chiancone and Gattoni, 1993). In most cases the effect is to reduce rather than eliminate the interaction, leading Davis and Williams (1998) to conclude that thermal denaturation alone may not be sufficient to dispel the allergic response, no doubt leading to the many studies on β-Lg conjugated to carbohydrates (e.g. de Luis *et al.*, 2007; Sperber *et al.*, 2009).

7.12 Function

The biological function of β-Lg remains elusive. The amino acid composition is such that the protein is certainly of nutritional value, but the molecular properties, particularly its resistance to acid and pepsin (Miranda and Pelissier, 1983; McAlpine and Sawyer, 1990; Guo *et al.*, 1995), and ligand binding lead to the supposition that some other, more specific, function exists. Further, the buried carboxyl, Glu_{89}, is strictly conserved, hinting at a general, gated ligand-binding activity. β-Lg is found to have bound fatty acids when separated under mild conditions (Diaz de Villegas *et al.*, 1987; Pérez *et al.*, 1989), and ligand binding increases the stability of the protein (Creamer, 1995; Shimoyamada *et al.*, 1996). Thus, might β-Lg function as an extracellular fatty-acid-binding protein passing on its cargo to the cytosolic form in the same way that is found for serum and cellular RBP? Unfortunately, this appears unlikely as neither porcine nor equine β-Lg binds fatty acids under physiological conditions, though β-Lg from all species binds retinol (Puyol *et al.*, 1991). Papiz *et al.* (1986) identified specific receptors in the intestine of the neonatal calf, suggesting a possible role in retinol transport or uptake, and to add weight to this idea, Said *et al.* (1989) have shown that β-Lg does enhance retinol uptake in the jejunum, and Puyol *et al.* (1995) have noted its β-Lg-assisted passage in cultured cells. Is β-Lg a facilitator of retinol uptake in the neonate? Wang *et al.* (1997) have pointed out that β-Lg binds vitamin D_2 more tightly than retinol. So might the role be that of a more general facilitator of vitamin uptake? Yang *et al.* (2009) suggest this, but their evidence is based upon a mouse model, and as mice do not produce β-Lg nor can they have encountered cows' milk during evolution, the suggestion is improbable.

A general function as inhibitor, modifier or enhancer of enzyme activity has been suggested (Farrell and Thompson, 1990; Pérez *et al.*, 1992; Pérez and Calvo, 1995). The protein phosphatase inhibition by β-Lg appears to be substrate-dependent and further, other milk proteins such as α-lactalbumin appear to have similar activity, so that the inhibition is probably not a genuine function. Enhancement of the activity of pregastric lipase (Perez *et al.*, 1992) also appears unlikely, since not every β-Lg binds fatty acids.

However, a function for the protein in the neonate may be illusory because β-Lg is not present in the milk of all species. Could the true function be associated with some process in the mother? The lipocalin sequence most closely related evolutionarily to β-Lg is glycodelin (Seppala *et al.*, 2009). What is more, the cladogram shown in Fig. 7.8 reveals that glycodelin is most closely related to baboon milk β-Lg. Glycodelin is a retinol-binding protein expressed in the first trimester of human pregnancy (Garde *et al.*, 1991), and retinol is an important modulator of differentiation (Evans and Kaye, 1999). The sequence of an equine endometrial RBP, p19, is available (Crossett *et al.*, 1996), but it is only distantly related to RBP, let alone the β-lactoglobulins and glycodelin. Thus, might β-Lg have evolved from an endometrial protein essential to the mother in early pregnancy but now mainly of nutritional

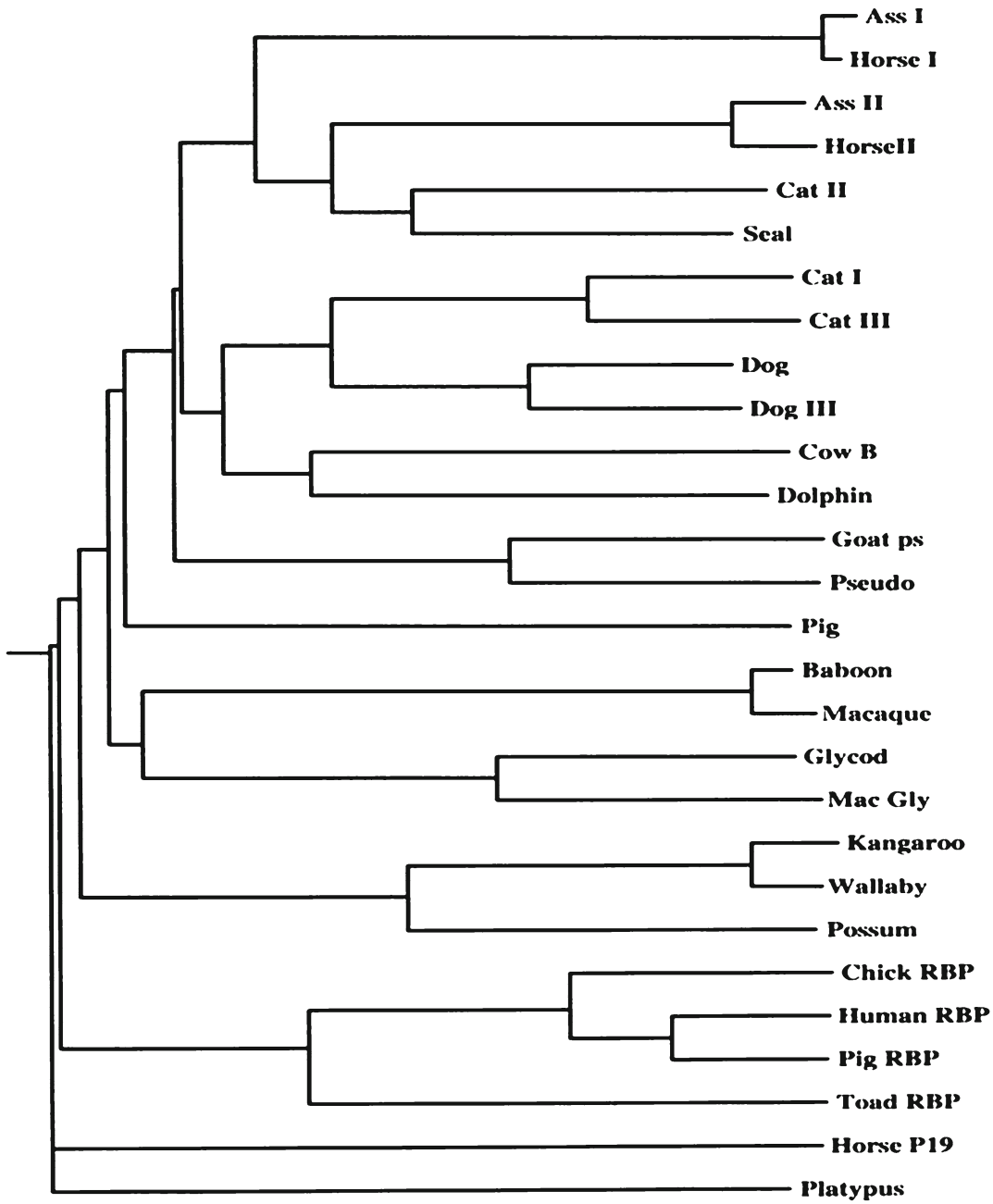

Fig. 7.8 The evolutionary relationship of β-lactoglobulins together with that of retinol-binding protein drawn by the PHYLIP server on the ExPasy server following a ClustalW alignment of the sequences. *Goat ps* goat β-Lg pseudogene; *Pseudo* bovine β-Lg pseudogene; *glycod* human glycodelin; *Mac Gly* macaque glycodelin. Horse P19 is an endometrial protein from the mare. The drawing is not to scale but shows that the glycodelins are more closely related to β-lactoglobulin than other lipocalins

value in the mammary secretion, although coincidental properties may also have arisen? The sequences of a glycodelin and a β-lactoglobulin from the same species do reveal a close relationship: of the 162 residues, 82 are identical with a further 28 similar. The presence of pseudogenes in the cow and goat that are relatively close to the cat II gene also leads one to ask if there are β-Lg pseudogenes in those mammals that do not express β-Lg in their milk. Indeed, there is reference to just such a pseudogene in the human genome (EMBL-Bank: AF403023-5) although there appears to be no further published information. However, this idea has a precedent since in ruminants, the whey acidic protein is present as a pseudogene, while the protein is expressed in porcine milk (Simpson *et al.*, 1998; Hajoubi *et al.*, 2006).

None of the above satisfactorily explains the strict conservation of Glu_{89} in the β-Lgs, though not in the glycodelins, where it is an Ala. The Glu_{89} is in a loop, and in the sequence alignment, an insertion is required nearby to optimise alignment with the marsupial proteins. Residue 91 in glycodelin is a Glu. More detailed study of the molecular properties of glycodelin than have been carried out so far would appear to be required. Also, if the proposed functional link is to be substantiated, the identification for β-Lg pseudogenes in rodents and rabbits is necessary, as is glycodelin in those species that produce β-Lg.

7.13 Conclusion

From the above somewhat selective review of the enormous literature on β-Lg, a view emerges of a molecule about which much is known. It is capable of binding small molecules and of interacting with large ones, including itself. Now that the protein is available in over-expressed forms in both yeast and *E. coli*, structure-based site-directed modifications that can affect these interactions are straightforward. Such changes to the protein have provided an explanation of the reversible conformational changes, much of the binding behaviour and self-association properties of the protein. In the next decade, they may even lead to a proper description of its biological function.

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α-Lactalbumin

8

K. Brew

8.1 Introduction

α-Lactalbumin (α-La) is uniquely expressed in the lactating mammary gland and is present in the milks of all major mammalian subdivisions, the eutherians, marsupials, and monotremes. Although first known as a component of the whey fraction of bovine and other milks, α-La was later found to be homologous with the type-c lysozymes and to have a primary function as the regulatory protein of lactose synthase (Brodbeck *et al*., 1967; Brew *et al*., 1967, 1968). The close similarity in 3D structure between α-La and lysozyme (Lz), initially predicted by homology-based model building (Browne *et al*., 1969), was confirmed when the crystallographic structure of baboon α-La was elucidated (Stuart *et al*., 1986; Smith *et al*., 1987; Acharya *et al*., 1989, 1990). The presence of a tightly bound calcium ion in α-La, initially discovered by Hiroaka *et al*. (1980), was found to involve a metal-binding site with a novel structure (Stuart *et al*., 1986) and further studies have shown that the calcium ion has a key role in structural stability and folding.

This account will include a synopsis of information discussed in the previous review in this series (Brew, 2003) but highlights recent findings. Much recent research has focused on partially folded forms of α-La in complexes with lipids that have apoptotic effects on cells, including tumor cells (Hakansson *et al.,* 1995, 1999). The biological significance and potential medical applications of these complexes are still being evaluated. α-La and Lz have been found to be members of a larger protein family that includes other mammalian proteins with possible functions in reproductive processes. The author apologizes to any investigators whose contributions have been inadvertently omitted.

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8.2 Overview of Earlier Work

8.2.1 Role in Lactose Biosynthesis

α-La is the regulatory protein of the lactose synthase enzyme system that catalyzes and regulates the synthesis of lactose in the lactating mammary gland (Fig. 8.1). The catalytic component of lactose synthase is a glycosyltransferase (GT), now known to be a member of CAZy (carbohydrate-active enzymes database) family 7 GTs (GT7; Campbell *et al*., 1997; Breton *et al*., 1998 Coutinho *et al*., 2003) which function in processing the glycans of glycoproteins and glycolipids. There are seven GT7 members in mammals, and the one responsible for lactose synthesis is designated UDP-galactose-*N*-acetylglucosamine β-1,4-galactosyltransferase-I (β-1,4-GT-I) (Lo *et al*., 1998). This enzyme was the

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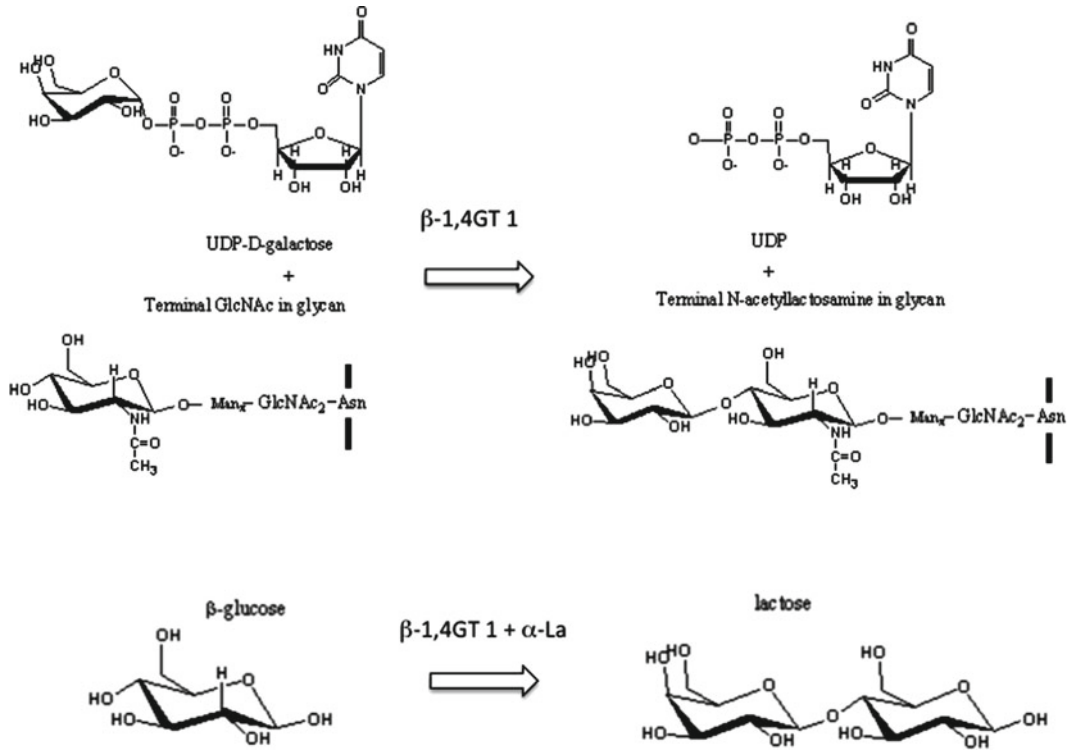

Fig. 8.1 The reactions catalyzed by β-1,4-GT-I: galactosyl of nonreducing terminal *N*-acetylglucosamine in *N*-linked glycoprotein glycans (in the absence of α-lactalbumin, α-La) and lactose synthesis (in the presence of α-La)

first GT7 to be cloned and sequenced (Shaper *et al*., 1986; Narimatsu *et al*., 1986); like most mammalian GTs that function in glycan synthesis and processing, β-1,4-GT-I is a type II membrane protein with a short N-terminal cytoplasmic domain, a transmembrane helix, a stem region, and a C-terminal catalytic domain. β-1,4-GT-I is expressed in various mammalian secretory cells, including mammary epithelial cells and normally catalyzes the transfer of galactose from UDP-galactose into a β-linkage with the 4-hydroxy group of β-linked *N*-acetylglucosamine in *N*-linked oligosaccharides in glycoproteins (Fig. 8.1).

The substrate specificity of β-1,4-GT-I is not stringent and it can, in isolation, catalyze lactose synthesis (Fig. 8.1) but inefficiently because of its remarkably low affinity for glucose (reflected in a K_M more than 1 M). α-La forms a 1:1 complex with β-1,4-GT-I synergistically with a glucose molecule; this reduces the K_M for glucose by about 1,000-fold, so that lactose synthesis can proceed efficiently at physiological concentrations of glucose. In contrast, α-La inhibits the binding of glycoprotein or oligosaccharide substrates to β-1,4-GT-I (Powell and Brew, 1976), effectively switching the acceptor substrate specificity of the enzyme from glycoproteins to glucose.

8.2.2 Organization and Regulation of Lactose Synthase

β-1,4-GT-I is a component of the membranes of the *trans*-Golgi and is retained in this compartment because of the length of its transmembrane helix (Masibay *et al*., 1993); its catalytic domain projects into the lumen of this Golgi. α-La, after synthesis in the endoplasmic reticulum is transported to the Golgi apparatus, where it interacts with the catalytic domain of the galactosyltransferase and promotes lactose synthesis; both α-La and lactose are secreted into milk via secretory vacuoles. The accumulation of lactose within the Golgi apparatus produces an osmotic flow of water into this compartment, a process that has an important role in the assembly of the aqueous

phase of milk (Brew, 2003). The organization of the system couples α-La production with lactose synthesis and milk secretion. Two research groups have generated mice in which the α-La gene is deleted and, also, in one case, replaced by a human α-La gene (Stinnakre *et al.,* 1994; Stacey *et al.,* 1995). Homozygous females with inactivated α-La genes produced small amounts of viscous milk containing no α-La or lactose and high levels of fat; these animals were unable to nourish their young. The presence of the human gene resulted in a high level of α-La expression and a small increase in milk volume. Other studies have shown that the concentration of lactose and milk production are enhanced in the milks of transgenic pigs that overexpress bovine α-La, resulting in increased growth rates in piglets (Wheeler, 2003).

8.3 Relationships with Lz and Other Proteins

The α-Las and type-c Lzs provide an example of extreme functional divergence in closely related proteins (Brew, 2003). Additional members of their gene family have now been identified in cDNA libraries from the human testis (Mandal *et al*., 2003; Chiu *et al*., 2004; Zhang *et al*., 2005) and were designated LYZL (lysozyme-like proteins): LYZL 2, 3, 4, and 6. LYLZ3 has also been called SPACA 3 (sperm acrosome associated 3; see Irwin *et al*., 2011); two of these proteins have the catalytic site Glu and Asp residues of the type-c lysozymes and may have Lz catalytic activity, but the others appear to have lost the Lz catalytic site and may be glycan-binding proteins (lectins). These proteins are principally expressed in the testis and, as indicated above, some are associated with the sperm acrosome. Based on their greater than 40% sequence identity to human Lz, they can be expected to have similar 3D structures to the α-Las and type-c Lzs. The genes for both human α-La and Lz are located on chromosome 12 (Davies *et al.,* 1987, Peters *et al*., 1989), and both contain four exons separated by three introns (Qasba and Safaya, 1984; Hall *et al.,* 1987; Vilotte *et al.,* 1987). Exon/intron boundaries are located at corresponding sites in known α-La and Lz genes. The genes for the other members of the LZLA family in humans are variously located on chromosomes 3, 10, 17, and X.

A few Lzs have been found to contain a high affinity calcium-binding site corresponding to that in α-La. Among them are Lzs from horse, donkey, dog, cat and echidna milks, and from pigeon eggs (Rodriguez *et al*. 1985; Nitta and Sugai, 1989; Godovac-Zimmermann *et al.,* 1988; Teahan *et al*., 1991; Tsuge *et al.,* 1992; Grobler *et al.,* 1994). Other Lzs with Ca^{2+}-binding sequence motifs include variants from zebra finch (*Taeniopygia guttata*), swimming crab (Pan *et al*. 2010), and some Lz variants from insects (Grunclova *et al*., 2003; Li *et al*., 2005). Earlier molecular phylogeny analyses indicated that the α-La and Lz gene lines separated prior to the divergence of the fishes and tetrapods (Prager and Wilson, 1988) and that Ca binding may have been an ancient feature of the Lz-α-La family that was lost from the "conventional" mammalian and avian Lz (Grobler *et al.,* 1994). This is supported by the phylogenetic tree shown in Fig. 8.3, which was produced (Dereeper *et al*., 2008) using the sequences shown in Fig. 8.2. A recent extensive investigation of the Lz family indicated that there are at least eight gene lines in mammals that developed prior to the divergence of the placental, marsupial, and monotreme groups. There are also multiple Lz relatives in nonmammalian vertebrates, including proteins that lack Lz catalytic residues, but other than the "standard" type-c Lz, none could be definitively identified as orthologs of members of the mammalian Lz family (Irwin *et al*., 2011).

An alignment of the amino acid sequence of human α-La with the sequences of α-Las from monotremes and a marsupial as well as homologues of Lz found in the human genome and representative Ca-binding lysozymes (a group that is not represented in the human genome) is shown in Fig. 8.2. Conserved residues in the whole group include eight cysteinyl residues that are structurally important since they form the four disulfide cross-links, some glycines and several other residues, that appear to be important for structure and/or stability (Brew, 2003). A comparison of the amino acid sequences of α-Las from different species shows that residues that are essential for

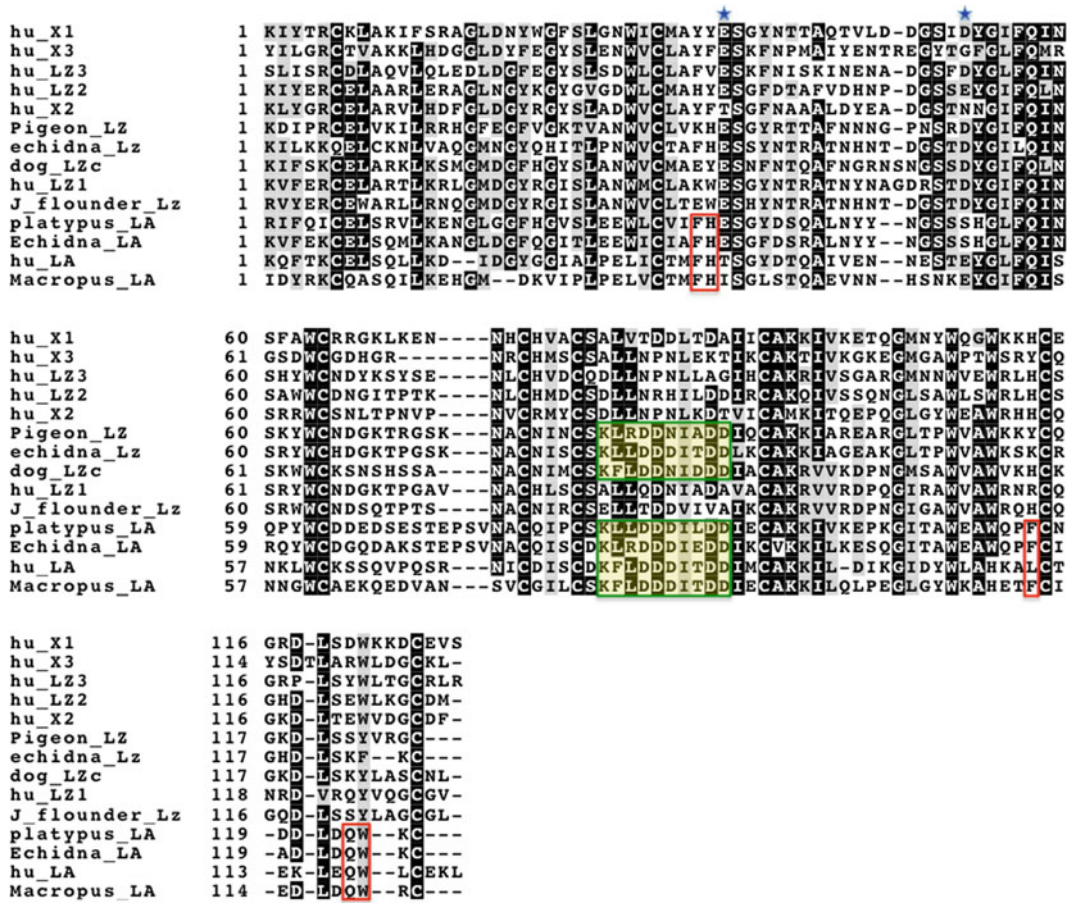

Fig. 8.2 An alignment of the sequences of selected representatives of different mammalian members of the lysozyme/α-lactalbumin family. Residues with shaded backgrounds are identical or chemically conserved in 70% of the sequences. The catalytic site Glu and Asp of the lysozymes are marked by blue stars and residues important for α-lactalbumin (α-La) activity are enclosed in *red boxes*. The Ca-binding sites in Ca-binding proteins are shaded in yellow. The proteins are hu_Lz1, human lysozyme (Lz); hu_Lz2, acrosome protein 5; hu_Lz3, Lz-like protein 6; Hu_X1, Lz-like protein 1; Hu_X2, acrosome protein 3; Hu_X3, Lz-like protein 4; Hu_LA, human α-La; Pigeon_Lz, Lz from *Columba livia* (Rock pigeon); echidna_Lz and Echidna_La, Lz and α-La from *Tachyglossus aculeatus*; J_flounder_Lz, Lz from *Paralichthys olivaceus* (Japanese flounder); dog_LZc, Ca-binding Lz from canine milk; platypus_LA, α-La from the duck-billed platypus; and Macropus_La, α-La from tammar wallaby (*Macropus eugenii*)

lactose synthase activity are conserved in α-Las from different species. However, there is some functional differentiation between species because α-Las from the distantly related monotremes have been found to be essentially inactive with eutherian galactosyltransferases, yet are active with monotreme galactosyltransferases (Shaw *et al.,* 1993; Messer *et al.,* 1997). This suggests mutual functional adaptation between the components of lactose synthase, but this has not yet been examined systematically.

Some marine mammals produce milk containing little or no lactose, but cetaceans (whales and dolphins) produce milk that contain lactose as the predominant sugar (Urashima *et al*. 2002, 2007). Parts of the amino acid sequence of α-La are known for many species in this order (Rychel *et al*., 2004), and all appear to differ from other

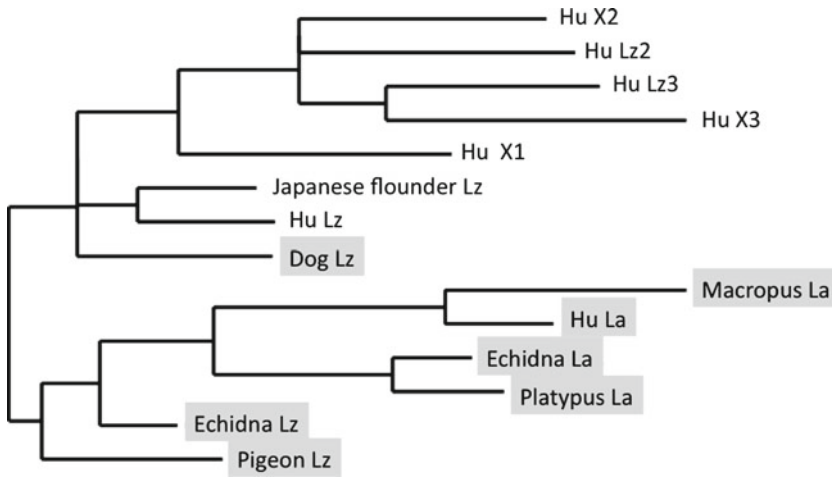

Fig. 8.3 A phylogenetic tree of members of the lysozyme (Lz)/α-lactalbumin (α–La) family. This was generated from the amino acid sequences shown in Fig. 8.2 using Phylogeny.fr (www.Phylogeny.fr; Dereeper *et al*., 2008). MUSCLE was used for aligning the sequences, Gblocks for curation, and MrBayes with 100,000 reiterations for constructing the tree. The tree was visualized using TreeDyn

known α-Las in having a unique unpaired cysteine at position 36. This could have a major effect on structure, stability, and/or activity but, at present, there are no reports regarding the properties of the α-La from these species.

8.4 Three-Dimensional Structures of Free and Complexed Forms of α-La

Overall structure: The crystallographic structures of free forms of an array of α-La variants have been determined including human, recombinant and natural bovine, goat, guinea pig, mouse, and buffalo (Acharya *et al*., 1991; Harata and Muraki, 1992; Ren *et al*., 1993; Pike *et al*., 1996; Calderone *et al*., 1996; Chandra *et al*., 1998; Chrysina *et al*., 2000). Also, the structure of mouse α-La has been elucidated in a variety of complexes with recombinant bovine β-1,4-GT-I and different ligands (reviewed by Qasba *et al*., 2008). The structure of α-La does not differ significantly between the free and complexed forms and is closely similar to those of the type-c Lzs. The dimensions of the α-La molecule are 23 Å×26 Å×40 Å and the structure includes three regular α-helices, two regions of 3_{10} helix, and a small three-stranded antiparallel β-pleated sheet separated by irregular β-turns (Fig. 8.4). Like Lz, α-La has a bilobal structure in which the α-helices form one lobe (or subdomain) and the small β-sheet and irregular structures the other (see Fig. 8.4).

The pairing of the eight cysteines of α-La to form four disulfide cross-links is identical to those in Lz, with bonds linking Cys_6 to Cys_{120} and Cys_{28} to Cys_{111}, in the helical lobe, a disulfide linking Cys_{60} to Cys_{77} in the β sheet-containing lobe and one between Cys_{73} of the β-lobe and Cys_{90} of the helical lobe. Crystallographic temperature factors (B factors) suggest that the C-terminal section of Lz has higher mobility than the rest of the molecule and this region appears to be even more dynamic in the α-La structure, displaying significantly different conformations in different α-La crystal structures, including structures of the same protein under different conditions. Residues 105–110, corresponding to the fourth helix in Lz, are helical in α-La structures determined at neutral or higher pH values but have a loop structure in crystals grown at lower pH values (Harata and Muraki, 1992; Pike *et al*., 1996). This appears to result from the protonation of His_{108} at the lower pH.

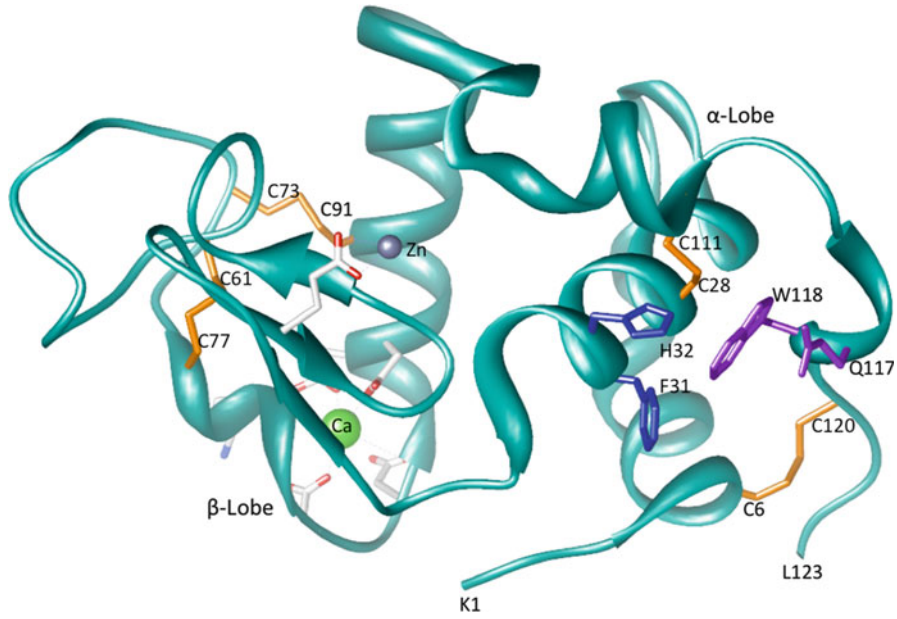

Fig. 8.4 The 3D structure of the Ca/Zn complex of human α-lactalbumin. The image was generated from Pdb file 1HML using Chimera (Pettersen *et al*. 2004). The side chains are shown for all eight cysteines and residues that are a key to its action in lactose synthase (Phe_{31}, His_{32}, Gln_{117}, and Trp_{118}) as well as residues in the Ca- and Zn-binding sites

Calcium-binding site: The Ca^{2+}-binding elbow (residues 79–88) is located at the junction of the two subdomains between a 3_{10} helix of the β-lobe (residues 76–82) and helix C (residues 85–93) of the α-lobe. The ion is coordinated by five oxygen atoms from side chain carboxyl groups of Asp_{82}, Asp_{87}, and Asp_{88} and peptide carbonyl oxygens of Lys_{79} and Asp_{84}. Two water molecules also coordinate the calcium, forming a slightly distorted bipyramid with liganding groups of the protein. The consensus amino acid sequence of this region in different α-Las is Lys_{79}-x-x-Asp-Asp-y-x-Asp_{88}, where x is a nonpolar amino acid and y a polar amino acid such as Asp, Glu, or Asn. An unusual feature of the binding site is the presence of four or five dicarboxylic residues within a 7-residue sequence. This pattern is conserved in the homologous Ca-binding Lzs (Brew, 2003).

Calcium is not required for the activity of α-La in lactose synthesis (Kronman *et al.,* 1981; Musci and Berliner, 1985) but strongly enhances the stability of the folded protein and is required for refolding and native disulfide bond formation in the reduced denatured protein (Rao and Brew, 1989). The effect of Ca^{2+} concentration on the rate of folding indicates that the increase in rate results from the binding of calcium to high-energy folding intermediates that have lower affinities for the metal ion than the native protein and therefore appear to have partially formed Ca^{2+}-binding sites (Kuwajima *et al.,* 1989; Kuwajima, 1996).

The crystallographic structures of the apo- and holo-forms of α-La (Chrysina *et al.,* 2000) have similar overall structures. Crystals of the apo-protein were grown at high ionic strength, but no cation or solvent molecules were present in the calcium-binding site and the structure of this part of the apo-protein is closely similar to that in the holo-protein. The main difference between the two structures is in the cleft region, close to Tyr_{103} and Gln_{54} on the opposite side of the molecule to the calcium-binding site. H-bonds between Tyr_{103} and groups in the α- and β-lobes are replaced by interactions with immobilized solvent molecules resulting in a more open cleft. It was proposed that the structural changes originate from charge repulsion between the negatively charged aspartic acid residues in the calcium-binding site; this

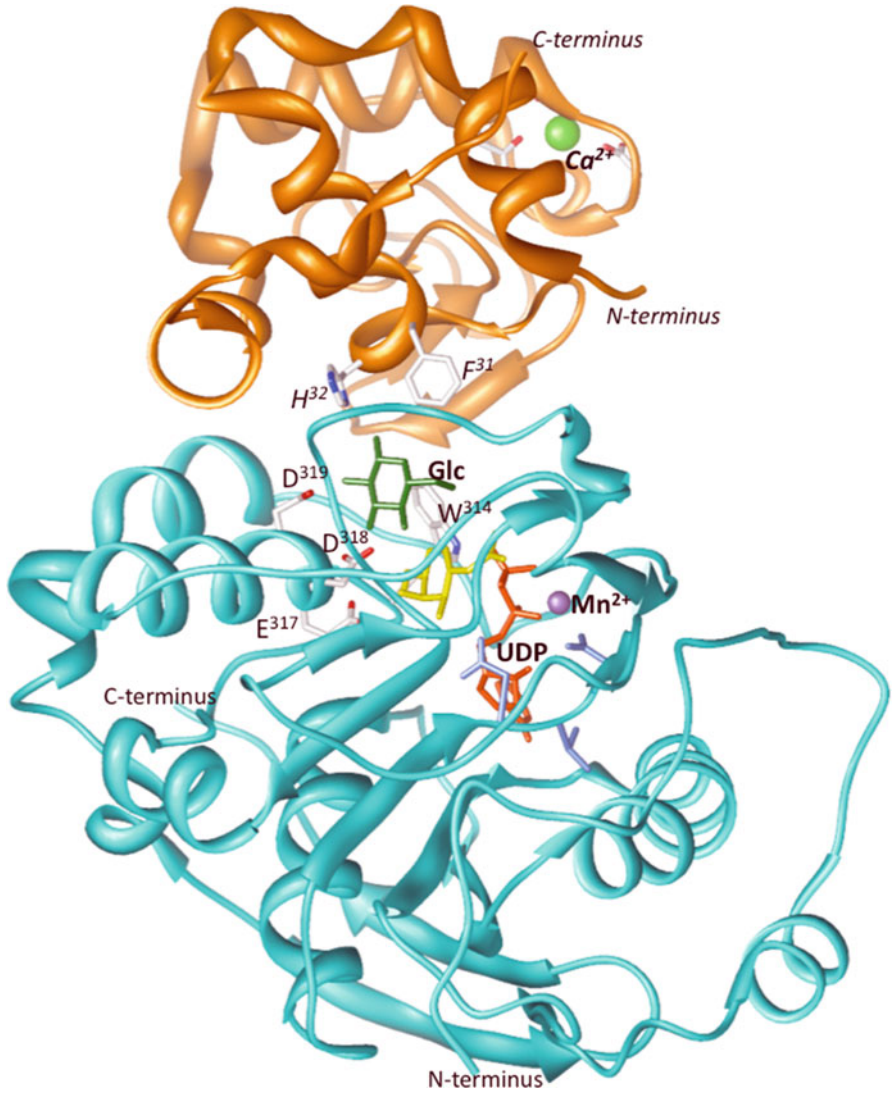

Fig. 8.5 Structure of the complex of the catalytic domain of bovine β-1, 4-GT-I (*cyan*) with mouse α-lactalbumin (α-La) (*orange*) together with glucose (*green*), Mn^{2+} (*purple*), UDP (*red*), and *N*-acetylgalactosamine (*yellow*). The image was generated from Pdb file 2 FYD using Chimera (Pettersen *et al*. 2004). Residues that have important roles in interactions between the proteins and with substrates are displayed and labeled, those from mouse α-La being *italicized*

generates a relatively small structural change in the binding site that affects the orientation of flanking secondary structure elements, 3_{10} helix h2 and α-helix C. These substructures are tethered by a disulfide bond (between Cys_{73} and Cys_{91}) and the slight expansion of the calcium-binding loop is transmitted to separate the α and β subdomains and perturb packing interactions in the hydrophobic box. The process may correspond to the initial step in the unfolding in α-La to the MG state and, in reverse, a mechanism for the effect of Ca^{2+} on the transformation of the molten globule (MG) to native state.

In apo α-La, at low ionic strength, the presence of a bound Ca ion will counter charge repulsion between the multiple carboxyl groups in the binding

site, explaining why the native structure of the apo-protein is unstable at low ionic strength so that the MG state becomes the predominant form (Kuwajima, 1989, 1996). The structure of the apo-protein shows that the absence of Ca, at high ionic strength, weakens interactions between the two lobes of the protein (Forge *et al*., 1999; Chrysina *et al*., 2000), which may be the basis for its large influence on folding kinetics in α-La.

Binding of other metals: The high-resolution structure (1.7 Å) for a complex of human α-La with Zn^{2+} and Ca^{2+} reveals that Ca^{2+} occupies the high affinity site, whereas Zn^{2+} binds in the cleft, coordinating with Glu_{49} from one molecule and Glu_{116} from a symmetry-related molecule, thus stabilizing an α-La dimer in the crystal (Fig. 8.3). The location of a second Ca-binding site was revealed by a 1.8 Å structure for human α-La in the presence of a high concentration (100 mM) of Ca^{2+} (Chandra *et al*., 1998). At this lower affinity site, the ion interacts with the side chain oxygens of Thr_{38}, Gln_{39}, and Asp_{83}, together with the carbonyl oxygen of Leu_{81}. Although Asp_{83} and Leu_{81} are components of the Ca-binding elbow, they are not involved directly in calcium binding at the primary site. The secondary Ca site appears to be similar to a binding site for Mn^{2+} (see Brew, 2003). Aramini *et al*. (1996) reported an NMR study of the binding of an array of metal ions to bovine, goat, and human α-La and concluded that most metal ions bind to the primary Ca^{2+} site with lanthanides and yttrium having the highest affinity ($Y^{3+} > La^{3+} = Lu^{3+} > Ca^{2+} > Sr^{2+} > Cd^{2+}, Pb^{2+}, Ba^{2+} > Sc^{3+}$). However, Co^{2+} and Cu^{2+} were found to bind at a different site, possibly corresponding to the zinc-binding site.

Complexes with β-1,4-GT-I: α-La forms a 1:1 complex with β4-GT-I in the presence of substrates, specifically glucose, *N*-acetylglucosamine or a combination of Mn^{2+}, and UDP-galactose (or other UDP-sugar). In isolation, β-1,4-GT-I is a poor catalyst for lactose synthesis because of its weak affinity for glucose (Brew, 2003) reflected in a K_M of more than 1 M. The synergistic binding of α-La and glucose to β-1,4-GT-I results in mutual stabilization of complexes and the 1,000-fold lowering of the K_M for glucose. α-La also enhances the affinity of GT for other monosaccharides, including xylose. In contrast, α-LA completes with disaccharide substrates such as diacetylchitobiose, or β-glycosides of GlcNAc, for binding to galactosyltransferase-I and consequently inhibits galactose transfer to such substrates.

The structures of several different complexes of mouse α-La with the catalytic domain of bovine β-1,4-GT-I have been determined by crystallography (Ramakrishnan and Qasba, 2001; Ramakrishnan *et al*., 2004, 2006; Qasba *et al*., 2008). β-1,4-GT-I has a GT-A fold, one of two predominant fold types (GT-A and GT-B) among GTs that utilize nucleotide-sugar substrates. In the GT-A fold, two Rossman-like α/β domains associate closely via a large interaction interface. The interaction interface between β-1,4-GT-I and α-La (lactose synthase) buries 1,310 $Å^2$ of accessible surface area from the two proteins, about 11% of the surface in β-1,4-GT-I and 20% of the accessible surface in the α-La molecule. Mutational studies of bovine α-La had previously shown that five residues have key roles, three (Phe_{31}, His_{32}, and Leu_{110}) by influencing glucose binding and two (Gln_{117} and Trp_{118}) by stabilizing the interactions of the two proteins (Malinovskii *et al*., 1996). The crystallographic structure in Fig. 8.3 shows that the residues in mouse α-La (Phe_{31}, His_{32}, Met_{110}, Gln_{117}, and Trp_{118}) corresponding to those identified by mutagenesis in bovine α-La are a key to its interactions with a largely nonpolar surface of β-1,4-GT-I containing Phe_{280}, Tyr_{286}, Gln_{288}, Tyr_{289}, Phe_{360}, and Ile_{363} and with glucose. An α-helix, containing residues 105–111 of α-La, interacts with a helix formed by residues 359–365 of the β-1,4-GT-I catalytic domain (Fig. 8.5). Conformational changes induced by substrate binding are features of the action of a wide array of GTs with GT-A folds and, in β-1,4-GT-I, the donor substrate (UDP-gal) stabilizes the complex with α-La by inducing a conformational change affecting residues 345–365. This results in α-helix formation by residues 359–365 and a change in positioning of Trp_{314}. When the acceptor substrate *N*-acetylglucosamine binds to β-1,4-GT-I, the 2-acetamido group binds to a hydrophobic pocket formed by Arg_{359}, Phe_{360}, and Ile_{363}. Glucose has a

hydroxy rather than acetamido at the 2-position and in the complex between β-1,4-GT-I, α-La, and glucose, α-La binds to this hydrophobic pocket and the Nδ1 of the imidazole group of α-La His32 interacts with the O-1 and O-2 hydroxy groups of glucose. The direct interaction of α-La with the 1-OH group of glucose explains how the binding of α-La and monosaccharides is synergistic, whereas α-La and oligosaccharide or glycoside acceptor substrates bind competitively to β-1,4-GT-I (Qasba *et al*., 2008).

8.5 Apoptotic Effects of α-La on Tumor and Other Cells

There is growing recognition that many proteins have multiple functions and much recent research on α-La has been focused on an activity that is distinct from its role in lactose synthesis and may be linked to its ability to form a stable partially folded structure, the MG. The MG state has a greatly diminished CD spectrum in the near-UV range, suggesting a disrupted tertiary structure, but a pronounced far-UV CD spectrum, indicating the presence of a high content of native secondary structure, particularly α-helices. α-La undergoes a transition to a MG state under mildly denaturing conditions such as acidic or alkaline pH, low concentrations of denaturants, or moderately elevated temperatures (see Kuwajima, 1989). In apo α-La the MG state is the predominant form at room temperature and low ionic strength. NMR studies (Rösner and Redfield, 2009) have shown that the MG form of α-La has a near-native α-subdomain whereas the smaller β-subdomain is unstructured.

A form of human α-La that has cytotoxic effects on tumor cells was initially isolated from acid-precipitated casein by Hakansson *et al.* (1995). Various methods have been reported for generating similar preparations, complexes of partially folded α-La with oleic acid, under more controlled conditions (Svensson *et al*., 1999, 2000, 2003; Permyakov *et al*., 2011); such preparations of human and bovine α-La were designated HAMLET (human α-lactalbumin made lethal to tumor cells) and BAMLET, respectively (Svensson *et al*., 2000; 2003). These preparations penetrate tumor and immature cells, disrupting mitochondria, nucleosomes, and proteosomes and activating apoptotic pathways (Mok *et al*., 2007; Mossberg *et al*., 2010).

There is considerable interest in potential clinical applications for HAMLET and similar preparations for cancer treatment and initial clinical trials on patients with skin papillomas and bladder cancer generated promising results (Gustafsson *et al*., 2004; Mossberg *et al*., 2007). However, a recent study indicates that some normal primary cell lines are more sensitive to α-La-oleic acid complexes than tumor cells and also that oleic acid alone has a cytotoxic action comparable to that of its complex with α-La (Brinkmann *et al*. 2011). Other work indicates that fully denatured bovine α-La-containing protein aggregates (as opposed to partially unfolded protein molecules) can be converted into cytotoxic complexes with oleic acid (Liskova *et al*., 2010) and apoptosis can be induced by oleic acid complexes with proteolytic fragments of bovine α-La (Tolin *et al*., 2010). These studies suggest that, although interesting and potentially significant, the nature and applicability of these α-La complexes need further evaluation.

While this work suggests a possible role for α-La in protecting suckling mammals from cancer, an alternative view of its biological significance has emerged from studies with marine mammals. In most mammals, mammary gland involution is linked to the accumulation of milk in the mammary ducts following the termination of suckling. This has been proposed to be triggered by a factor or factors in the milk that accumulates after weaning. Female Cape fur seals have an unusual lactation pattern; they intensively feed their pups for 2–3 days on land with copious quantities of rich milk that is high in protein and lipid but devoid of lactose. Subsequently they go on extended foraging trips lasting up to 23 days. In most lactating mammals, if milk is not removed for 23 days, mammary cell apoptosis and involution ensue, but this does not

occur in the fur seal. In this species, the α-La gene is altered so that little or no protein is produced. Also, the protein product is truncated relative to the α-Las from other species, with 104 rather than 123 residues, and lacks parts of the structure that are essential for its activity in lactose synthase and for formation of a stable molten globule structure (Reich and Arnould, 2007; Sharp *et al*., 2008). These changes have been proposed to prevent apoptosis triggered by α-La complexes so that mammary function can be retained despite prolonged intervals between suckling activities (Sharp *et al*., 2008). This might be interpreted to suggest an interesting symmetry in the role of α-La in lactation as a terminator as well as an initiator and maintainer of lactose synthesis. However, this is speculative and it is possible that the example of the fur seal may be a unique adaptation.

8.6 Conclusions

The structural basis of the ability of α-La to regulate substrate specificity in β-1,4-GT-I is now understood in detail but the evolutionary relationship between α-La and calcium binding and "conventional" Lz has become complicated by the identification of additional members of the Lz family, including some with non-catalytic activities (Fig. 8.2). It seems likely that the α-La gene developed at an early time prior to the origins of the synapsids, possibly contributing small amounts of lactose to a secretion from apocrine-like glands that had a role in supplying antimicrobial oligosaccharides and fluids to thin-shelled eggs (Oftedal, 2002). The significance of lactose seems likely to have been twofold: (a) increasing the volume of secreted fluids through osmotic effects and (b) serving as a core or primer for the synthesis of larger complex oligosaccharides. The role of lactose as a nutrient for young animals may have developed later as it was produced in larger quantities as the efficiency of the lactose synthase system increased during the course of evolution (Capuco and Akers, 2009). As discussed in Sect. 4, the ability of α-La to undergo conversion to a pro-apoptotic complex with oleic acid or other lipids is interesting, but there seem to be some issues regarding specificity. Thus, recent studies have shown that the Ca-binding Lz from equine milk can also undergo a conformational change and form complexes with oleic acid that have cytotoxic effects on cells (Wilhelm *et al*., 2009; Nielsen *et al*. 2010). Whether these protein complexes are vehicles that merely deliver oleic acid to a membrane or whether the protein component has a specific role in cell-specificity or apoptosis remains to be determined. The possible role of α-La, and perhaps Ca-binding milk Lzs, in mammary involution (Sharp *et al*., 2008) is also intriguing and merits further investigation.

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Immunoglobulins in Mammary Secretions

9

W.L. Hurley and P.K. Theil

9.1 Introduction

Immunoglobulins (Igs) secreted in colostrum and milk are a major factor providing immune protection to the neonate. The Igs in milk represent the cumulative immune response of the lactating mammal to exposure to pathogens and other sources of antigenic stimulation that occurs through interaction with the environment. Extensive species variability exists on how and when the Igs are transferred to the neonate as well as on the mechanisms by which the Ig impacts the neonate (Butler and Kehrli, 2005). While colostrum and milk Igs have been a topic of study since the late nineteenth century, herdsmen have capitalized on the value of colostrum and milk immune factors for the neonate for many centuries (Larson, 1992; Wheeler *et al.*, 2007). The Igs found in colostrum and milk and the role in transfer of passive immunity from mother to neonate have been reviewed by many authors (Brambell, 1970; Butler, 1974, 1983; McClelland, 1982; Chernishov and Slukvin, 1990; Larson, 1992; Telemo and Hanson, 1996; Korhonen *et al.*, 2000a; Hanson *et al.*, 2001; Lilius and Marnila, 2001; Tizard, 2001; Uruakpa *et al.*, 2002; Hurley, 2003; van de Perre, 2003; Butler and Kehrli, 2005; Wheeler *et al.*, 2007; Gapper *et al.*, 2007; Stelwagen *et al.*, 2009; Brandtzaeg, 2010; Hurley and Theil, 2011). This chapter reviews the Igs found in mammary secretions in the context of their diversity of structure, origin, transfer, and function.

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9.2 The Immunoglobulins

9.2.1 Classes and Structure of Immunoglobulins

Immunoglobulins found in colostrum or milk are the same as those found in the blood or mucosal secretions. They are a family of proteins with a range of protective bioactivities. Immunoglobulin synthesis occurs through a complex process of gene rearrangement and combinatorial joining of gene segments, addition or removal of nucleotides at the point of joining (junctional diversity), and somatic hypermutation of variable region gene segments (Butler, 1998; Marchalonis *et al.*, 1998; Schlissel, 2003; Maul and Gearhart, 2010; Chrony *et al*. 2010). An antibody repertoire of greater than 10^{12} may be expected (Butler, 1998; Moser and Leo, 2010); however, variability exists among species on how these mechanisms of creating antibody diversity are employed (Meyer *et al.*, 1997; Butler, 1998; Marchalonis *et al.*, 1998; Schlissel, 2003; Maul and Gearhart, 2010; Chrony *et al*. 2010).

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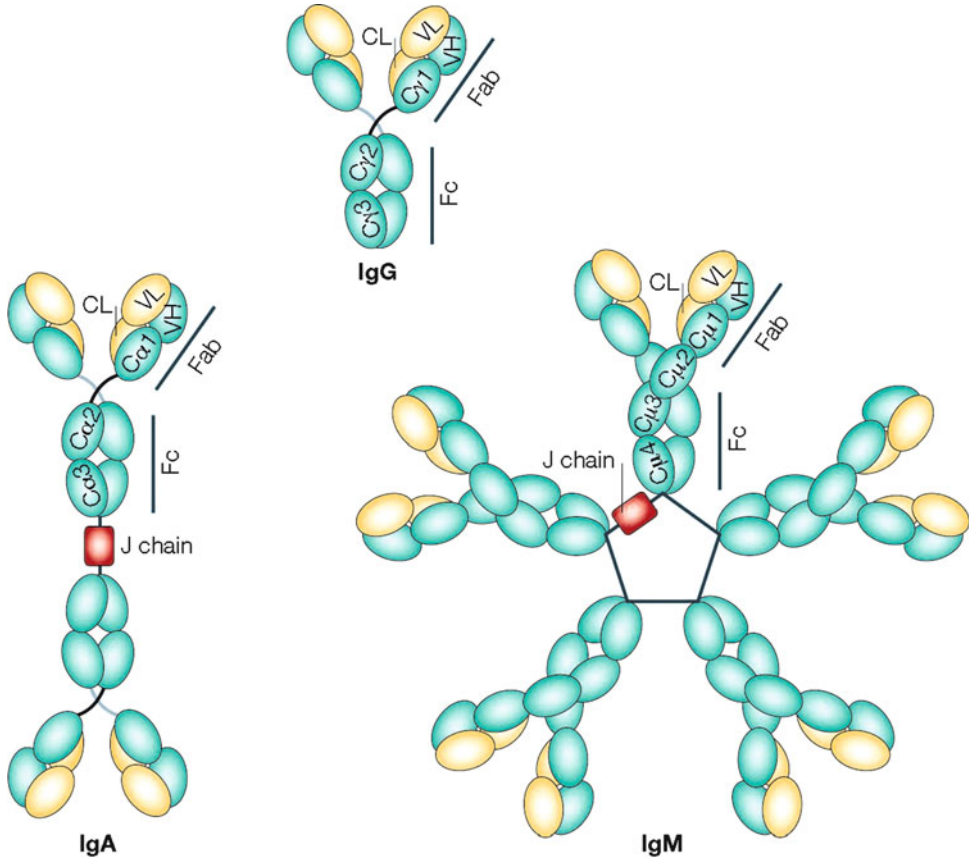

Fig. 9.1 Immunoglobulins are Y-shaped heteromeric complexes composed of two light chains (~25 kD) and two heavy chains (~55 kD) in the case of IgG. The light chains interact with the amino terminus of the heavy chains to form the Fab domain of the molecule which contains the antigen-binding sites at their tips. The carboxyl portion of the heavy chains combines to form the Fc domain which allows for interactions with complement and Fc receptors. Immunoglobulin classes are distinguished by the type of heavy chain and their ability to interact with the J or joining chain (~15 kDa) which links the heavy chains to form polymeric immunoglobulins in the case of IgA and IgM (Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, 3:1–12, 2002)

Mammalian antibodies can be divided into five classes or isotypes, IgG, IgA, IgM, IgE, and IgD. All monomeric Ig molecules consist of a similar basic structure composed of four subunit polypeptides, including two identical heavy chains and two identical light chains, with a total molecular mass of ~160 kDa. Heavy and light chains are both composed of domains referred to as variable (V_H, V_L) and constant (C_H, C_L) regions. Disulfide bonds link each heavy and light chain pair, as well as link the two heavy chains, resulting in a Y-shape molecule with two antigen-binding sites (Fig. 9.1). The number and position of disulfide bonds linking heavy chains varies with Ig isotype. The characteristic Y-shape of immunoglobulins is recognizable even with electron microscopic analysis (Roux, 1999). Physicochemical properties of immunoglobulins found in bovine milk have been summarized by Larson (1992).

The N-terminal portion of the Ig molecule is the antigen-binding region (Fig. 9.1). Antigen binding occurs through interactions of the antigen with the variable regions of heavy and light chains. Digestion of the IgG molecule with papain hydrolyzes the heavy chain at the hinge region and releases two identical antigen-binding fragments (Fab) and the constant portion of the molecule (Fc). The Fab consists of V_H and C_{H1} domains of the heavy chain and V_L and C_L domains of the light chain. The Fc portion of the IgG molecule consists

of the C_{H2} and C_{H3} domains. Upon digestion of IgG by the stomach proteolytic enzyme, pepsin, a $F(ab')_2$ fragment is produced which includes the two antigen-binding (Fab) sites of the IgG molecule (Nisonoff *et al*. 1960; Fang and Mukkur, 1976; Butler, 1983; Mix *et al.*, 2006).

The Fc fragment contains the portion responsible for many of the biological activities of the antibody molecule, including complement activation, recognition by Fc receptors on leukocytes and epithelial cells, transport through epithelial cells, and recognition by bacterial Ig-binding proteins. *N*-linked glycosylation of the Fc portion of IgG is thought to keep the heavy chains in an open conformation, contributing to the binding of the Fc to Fcγ receptors (FcγR; Anthony and Ravetch, 2010). Binding of Ig Fc to FcγR occurs asymmetrically, resulting in a 1:1 receptor:ligand stoichiometry (Radaev and Sun, 2001).

9.2.2 Immunoglobulins in Biological Fluids

Immunoglobulin G, IgA, and IgM are the major Ig isotypes in mammary secretions. Physical and biochemical properties of Igs of bovine mammary secretions have been described previously (Butler, 1983, 1986; Eigel *et al.*, 1984; Larson, 1992). Immunoglobulin G exists in the monomeric form in blood or milk. Most serum IgA is monomeric (Mestecky *et al.*, 1999), while most IgA in mucosal secretions are di- or tetrameric IgA. In the polymeric form of IgA, the monomers are linked together near the C-terminal of the heavy chains through covalent interaction with the J or joining chain (~15 kDa; Fig. 9.1) (Brandtzaeg, 1985). The mass of dimeric IgA, including the J chain, is ~370 kDa. Serum and milk IgM are complex molecules composed of five IgM monomers linked by disulfide bonds and containing one J chain and having a molecular mass of ~1,000 kDa (Fig. 9.1). The polymeric nature of IgA and IgM and their binding to the J chain give them a high valency of antigen-binding sites and the ability to agglutinate bacteria, as well as a limited complement-activating activity which allows them to act in a noninflammatory manner, and a high affinity for the polymeric immunoglobulin receptor (pIgR) that is responsible for transepithelial transport of IgA and IgM into mucosal secretions (Johansen *et al.*, 2000, 2001; Braathen *et al.*, 2007).

The predominant Ig in colostrum varies among species and is related to the route of transfer of passive immunity from mother to offspring. Concentrations of IgG are greatest in the colostrum of ruminants and other ungulate species (Table 9.1, Fig. 9.2). Highest concentrations of Igs in bovine mammary secretions are found in colostrum removed immediately after parturition (Guidry *et al.*, 1980; Larson *et al.*, 1980; Larson, 1992). The total quantity of IgG1 secreted by the mammary gland of the dairy cow during the peripartum period can exceed 2 kg, resulting in a reduction of the concentration of IgG1 in the maternal blood serum (Larson *et al.*, 1980; Larson, 1992). Estimates of concentrations of Ig in colostrum and milk are variable and can be affected by parity, genetics, stage of lactation, and management of the animal (Newstead, 1976; Oyeniyi and Hunter, 1978; Guidry *et al.*, 1980; Muller and Ellinger, 1981; Norman and Hohenboken, 1981; Devery-Pocius and Larson, 1983; Guidry and Miller, 1986; Caffin and Poutrel, 1988; Gilbert *et al.*, 1988; Pritchett *et al.*, 1991; Quigley *et al.*, 1994).

The primary Ig isotype in human colostrum and milk is IgA (Table 9.1, Fig. 9.2). Combined with high concentrations of lactoferrin (Chap. 10) and high activity of lysozyme, human milk has a particularly high antimicrobial activity (Goldman, 1993; Xanthou *et al.*, 1995; Goldman and Ogra, 1999). Immunoglobulin G seems to be the major colostrum isotype in rat colostrum (Table 9.1), which is consistent with the much studied specific intestinal absorption of IgG by the neonatal rat intestine (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985).

9.2.3 Properties of Immunoglobulins in Mammary Secretions

When milk is incubated with radiolabeled bovine Ig and components are separated by ultracentrifugation, >90, 85, 80, and 70% of the IgG1, IgG2, IgA, and IgM, respectively, are found in the whey fraction (Frenyo *et al.*, 1986). The fat (cream) fraction does contain a portion of the Ig, with the

Table 9.1 Concentration of immunoglobulins and percentage of major component in serum and mammary secretions of several speciesa (Adapted from Larson (1992))

		Concentration (mg/mL)			Major component Immunoglobulin (%)		
Species	Immunoglobulin	Blood serum	Colostrum	Milk	Serum	Colostrum	Milk
Cow (*Bos taurus*)	IgG-total	2,500	32–212	0072	88	85	66
	IgG_1	1,400	20–200	006			
	IgG_2	1,100	1,200	0012			
	IgA	004	305	0013			
	IgM	301	807	0004			
	FSC		005	002			
Horse	IgG-total	2,109	11,304	0039	89	88	43
	IgG(T)	802	1,502	0009			
	IgA	105	1,007	0048			
	IgM	102	504	0003			
Pig	IgG	2,105	5,807	300	89	80	70
	IgA	108	1,007	707			
	IgM	101	302	003			
Dog[b]	IgG	1,101	2,304	0024	81	68	85
	IgA	007	908	2,063			
	IgM	107	008	0022			
Rat[c]	IgG-total	2,406	206	—[d]	96	76	—[d]
	IgG_{2a}	800	009	1,053			
	IgA	0015	008	0059			
	IgM	0077	ND[e]	ND[e]			
Human	IgG	1,201	0043	0004	78	90	87
	IgA	205	17,035	1,000			
	IgM	0093	1,059	0010			
	FSC		2,009	0002			

[a]Approximate values, some from limited observations. Data compiled and calculated from human and pig (Butler, 1974), rat (Stechschulte and Austen, 1970; Bazin *et al*., 1974; McGhee *et al*., 1975; Michalek *et al*., 1975; Rousseaux and Bazin, 1979), dog (Vaerman and Heremans, 1969; Heddle and Rowley, 1975), horse (Rouse and Ingram, 1970; Vaerman *et al*., 1971; McGuire and Crawford, 1972), cow (Butler, 1981, 1983; Devery-Pocius and Larson, 1983) Certain IgG subclasses for rat, horse, and cow are shown and included in the total IgG. Where subclasses in other species were reported, they are grouped in the total for the class

[b]See Larson (1992) for discussion of dog colostral Ig concentration reported by others

[c]Data for the rat are inconsistent. Values given are average from several studies. See Larson (1992)

[d]Total IgG estimated to be >1,053 mg/mL and 72% (Larson, 1992)

[e]*ND* Not consistently detected

prevalence of the IgM and IgA in the cream greater than that for the IgG1 and IgG2. The casein pellet also contains a small fraction of the IgM and IgG2.

Concentrated Ig in bovine colostrum is rather stable at refrigerated temperatures or frozen. This has practical value to the dairy industry for the storage of colostrum containing high Ig concentrations for feeding newborn calves. On the other hand, Ig is heat-labile (Goldsmith *et al.*, 1983; Calmettes *et al.*, 1991; Larson, 1992; Fukumoto *et al.*, 1994; Lindstrom *et al.*, 1994; Dominguez *et al.*, 1997; Mainer *et al.*, 1997; Chen and Chang, 1998; Chen *et al.*, 2000). This is of particular importance where colostrum or milk is pasteurized for use to treat or control disease (Godden *et al.*, 2003, 2006; McMartin *et al.*, 2006; Elizondo-Salazar *et al.*, 2010). Heat denaturation results in conformational changes in the Ig molecule (Calmettes *et al.*, 1991), and particularly in the antigen-binding activity of the Ig (Dominguez *et al.*, 1997, 2001), which is more thermolabile than the other regions of the molecule (Mainer *et al.*, 1997; Dominguez *et al.*, 2001).

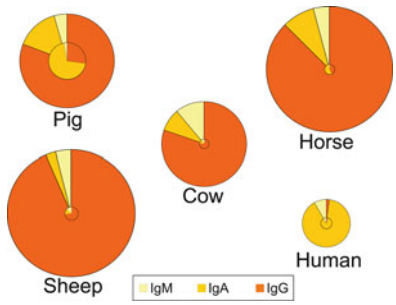

Fig. 9.2 Relative distribution of IgG, IgA, and IgM in colostrum (*outer circle*) and in milk (*inner circle*) of five species. The relative size of the circles represents the overall concentration of total immunoglobulins found among the species and the concentrations in colostrum vs. milk. Data compiled and calculated from cow and sheep (Butler and Kehrli, 2005), human and pig (Butler, 1974), and horse (Rouse and Ingram, 1970). From Hurley and Theil (2011), Open Access, MDPI Publishing, Basel, Switzerland

Immunoglobulin G is the most thermostable and IgM is the most thermolabile of the Ig found in bovine milk (Mainer *et al.*, 1997). Milk samples that undergo typical pasteurization can retain 25–75% of the IgG concentration; however, milk undergoing ultrahigh temperature pasteurization retains little detectable IgG (Li-Chan *et al.*, 1995). Several alternative methods for microbial inactivation of milk have been developed which may avoid the effects of heat treatment on Ig solutions (reviewed in Hurley and Theil, 2011).

Isolated bovine milk IgG, which is stable for several hours at 37°C when at pH 6 to 7, has significantly reduced stability when at pH ≤3 or at pH ≥10 (Shimizu *et al.*, 1993; Chen and Chang, 1998). Elevated temperature conditions enhance the negative effect of pH on IgG stability (Dominguez *et al.*, 2001; Gao *et al.*, 2010).

9.3 Origins of Immunoglobulins in Mammary Secretions

9.3.1 Sources of Immunoglobulins in Mammary Secretions

Immunoglobulins found in mammary secretions arise from systemic and intramammary origins. The proportion of colostrum IgG that is produced by plasma cells in the mammary gland is minor compared with that which is absorbed from the serum. Much of the IgA and IgM found in colostrum and milk is produced by plasma cells in the mammary tissue. Mammary gland plasma cells lie adjacent to the mammary alveolar epithelial cells (Nickerson and Heald, 1982; Sordillo and Nickerson, 1988). Bovine mammary tissue contains plasma cells producing IgG, IgA, and IgM isotypes, with IgG-producing cells predominating during lactation and involution (Yurchak *et al.*, 1971; Sordillo and Nickerson, 1988).

Mammary plasma cells arise from migration of lymphocytes from the gut-associated lymphoid tissue (GALT), which includes the Peyer's patches, lymphoid and myeloid cells in the *lamina propria*, and intraepithelial lymphocytes (Husband, 1985; Hunziker and Kraehenbuhl, 1998; Kelsall and Strober, 1999; Ishikawa *et al.*, 2005; Spenser *et al.*, 2007). Maternal exposure to antigens through the gastrointestinal tract results in activation of GALT lymphocytes. These GALT lymphocytes reflect the antigen exposure response in the mother's mucosal immune system and provide a direct link between intestinal and mammary immune systems (Telemo and Hanson, 1996; Hunziker and Kraehenbuhl, 1998; Brandtzaeg, 2010). As a consequence, maternal colostrum and milk contain antibodies specific for pathogens that may be encountered by the neonate's intestine and other mucosal tissues (Hanson *et al.*, 2001; Brandtzaeg, 2003, 2010).

9.3.2 Mammary Gland Transport of Immunoglobulins

Transepithelial transport of Ig occurs through a mechanism where the Fc portion of the Ig molecule binds to the Fc receptor at the basolateral surface of the cell (Larson, 1992; Hunziker and Kraehenbuhl, 1998; Cianga *et al.*, 1999; Kacskovics, 2004; Butler and Kehrli, 2005), or binding to the receptor may occur once the Ig is internalized *via* endocytosis (Cervenak and Kacskovics, 2009). The receptor-bound immunoglobulin then is internalized into the cell through an endocytic mechanism, transported to the apical end of the cell, and released into the alveolar lumen (He *et al.*, 2008; Cervenak and Kacskovics, 2009).

9.3.2.1 IgG

In the mammary gland, IgG is thought to be transported across the epithelial cells by the Fc receptor known as FcRn, or the neonatal Fc receptor. This Fc receptor was initially identified as the receptor in the intestine of the neonatal rodent that was responsible for the specific uptake of maternal IgG (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985). The FcRn also has been implicated in the transplacental transport of IgG in humans and other species (Simister and Story, 1997; Simister, 2003; Fuchs and Ellinger, 2004; Pentsuk and van der Laan, 2009) as well as being described in a range of other tissues (Cervenak and Kacskovics, 2009). The FcRn is a heterodimer. The MHC class I protein, ß$_2$-microglobulin, is the smaller subunit (Simister and Mostov, 1989; Hunziker and Kraehenbuhl, 1998). It has a monomeric molecular mass of ~12 kD, but exists as a tetramer in milk (Whitney, 1988). Free bovine milk β_2-microglobulin may arise from milk monocytes (Pringnitz *et al.*, 1985a, b). The larger subunit of FcRn is an integral membrane protein structurally related to MHC class I α chains (Simister and Mostov, 1989; Burmeister *et al.*, 1994; Ghetie and Ward, 1997). Milk from mice in which the β_2-microglobulin gene has been deleted still has normal concentrations of IgG (Velin *et al.*, 1996). Binding of IgG to FcRn is pH-dependent. Binding occurs with high affinity at acidic pH, while only weak binding occurs at neutral or basic pH (Cervenak and Kacskovics, 2009), perhaps indicating that binding of IgG to FcRn in epithelial cells may occur within the endosome's acidic environment.

Mammary epithelial cells rapidly take up IgG1 at their basolateral membrane surface during colostrum formation, and large amounts of IgG1 can be observed both in the cells and accumulated in the lumen (Leary *et al.*, 1982; Larson, 1985). Binding of IgG1 to receptors on epithelial cells also might be responsible for the low concentrations of the IgG found in cows' milk during lactation (Sasaki *et al.*, 1977); however, mammary tissue leucocytes also contribute to IgG1 binding in the tissue (Barrington *et al.*, 1997a).

Immunoglobulin G1 and IgG2 are present in approximately equal concentrations in serum. In contrast, IgG1 is present in colostrum and milk at a substantially higher concentration than IgG2 (Guidry *et al.*, 1980). There appears to be preferential transport of IgG1 into the mammary secretions (Sasaki *et al.*, 1977). Interestingly, IgG2 appears to have a higher affinity for FcRn than IgG1 (Cervenak and Kacskovics, 2009). If FcRn is responsible for IgG transport across the epithelial cell during colostrum formation, then how can the higher transport of IgG1 occur in the face of the higher affinity for IgG2? One explanation may be found in the proposed role of FcRn in the recycling of IgG in various tissues (Junghans and Anderson, 1996; Junghans, 1997; Telleman and Junghans, 2000). The loss of IgG through various tissues normally may be minimized by IgG binding to FcRn in the cells and being recycled back to the blood or lymph. Overexpression of FcRn in transgenic mice results in an extended serum IgG half-life (Bender *et al.*, 2007; Lu *et al.*, 2007). This recycling function of FcRn, with the higher affinity for IgG2, may suggest that IgG2 taken up by the mammary epithelial cell during colostrum formation is preferentially recycled back to the extracellular fluid and not passed on to the alveolar lumen, resulting in the apparent preferential transport of IgG1 into the mammary secretion. This mechanism does not account for the apparent higher affinity of binding for IgG1 compared with IgG2 described in collagenase-dispersed cell cultures from prepartum bovine mammary tissue (Sasaki *et al.*, 1977).

Genetic variants of the gene coding for the MHC class I α chain of FcRn (*FCGRT*) are associated with IgG concentration in colostrum of dairy cows (Zhang *et al.*, 2009). A genetic or hormonal component to the regulation of Ig transport may account for part of the variance in mass transfer of IgG1 into colostrum in dairy cattle (Baumrucker *et al.*, 2009). These observations suggest an opportunity to enhance the concentrations of Ig in colostrum and milk through genetic manipulation.

9.3.2.2 Secretory IgA and IgM

Secretory IgA generally is the major colostral and milk Ig in species where IgG transport occurs during gestation (Table 9.1). Transepithelial

transport of dimeric IgA and pentameric IgM occurs *via* the transmembrane glycoprotein polymeric immunoglobulin receptor, or pIgR (Mostov and Kaetzel, 1999; Kaetzel and Bruno, 2007). The polymeric nature of IgA and IgM arises from their binding with the J-chain peptide (Johansen *et al.*, 2000), which in turn results in their high affinity for pIgR (Johansen *et al.*, 2000, 2001; Braathen *et al.*, 2007). The pIgR binds dimeric IgA or pentameric IgM at the basolateral membrane of the cell (Mostov, 1994; Morton *et al.*, 1996; Raghavan and Bjorkman, 1996; Mostov and Kaetzel, 1999). The pIgR-IgA or -IgM complexes translocate through the mammary secretory cell by an endocytic process to the apical surface (Hunziker and Kraehenbuhl, 1998; Mostov and Kaetzel, 1999). The pIgR is hydrolyzed to release secretory component (SC; ~75 kDa), the receptor fragment that remains bound to the Ig molecule (Hunziker and Kraehenbuhl, 1998; Mostov and Kaetzel, 1999). Receptor sites not occupied by Ig also are hydrolyzed to release free SC, which potentially may neutralize the effect of several pathogens (Brandtzaeg, 2003). Free SC is present in colostrum and milk (Pringnitz *et al.*, 1985a, b). The ratio of dimeric to tetrameric sIgA in milk and saliva is about 3:2 (Mestecky *et al.*, 1999), while monomeric IgA in milk and saliva represents about 5–10% of total IgA, respectively.

9.3.3 Control of Transport and Mammary Gland Immunity

Transepithelial transport of Ig in the mammary gland occurs in relation to the physiological state of the mammary tissue. A role for ovarian steroid hormones in stimulating selective transport of IgG in the bovine mammary gland was demonstrated originally when treatment of non-lactating cows with estrogen and progesterone resulted in the formation of colostrum (Smith *et al.*, 1971). This observation has provided the basis for many subsequent efforts to hormonally induce lactation in cattle. Lactogenic hormones generally decrease the transport of IgG in the mammary gland (Winger *et al.*, 1995; Barrington *et al.*, 1997b). Prepartum removal of mammary secretions in cattle can alter the concentration of IgG1 in secretions (Guy *et al.*, 1994). The effect of prepartum unilateral removal of mammary secretions on secretion composition suggests that local mammary gland factors also affect IgG1 transport (Guy *et al.*, 1994). Both hormonal and local factors contribute to the control of IgG1 transport in the ruminant mammary gland (McFadden *et al.*, 1997). Selective transfer of IgG1 into milk occurs during mammary gland inflammation (Darton and McDowell, 1980), resulting in acute increases in the concentration of Ig in milk during mastitis (Harmon *et al.*, 1976; Guidry and Miller, 1986; Caffin and Poutrel, 1988).

Expression of the pIgR in rabbit mammary tissue is inhibited by elevated progesterone and estrogen concentrations, but is stimulated by prolactin (Rosato *et al.*, 1995). This is consistent with the prepartum increase in mammary tissue IgA transport and pIgR expression (Rosato *et al.*, 1995). Expression of pIgR in the mammary gland of the ewe also appears to be under the control of hormones responsible for initiation of lactation (Rincheval-Arnold *et al.*, 2002). Expression of pIgR also may be regulated by cytokines (Hunziker and Kraehenbuhl, 1998).

9.4 Transfer of Passive Immunity

9.4.1 Mother to Neonate

The mammalian neonate's immune system develops slowly and initially is dependent upon maternal antibodies to provide disease protection. Mechanisms of transport of passive immunity from mother to neonate vary among mammalian species (see Butler and Kehrli, 2005). The neonate of ungulate species is born essentially agammaglobulinemic and requires absorption of a substantial mass of maternal antibody from colostrum to attain sufficient systemic immunity to protect from disease during early postnatal development. In these species, IgG1 is typically the major Ig found in colostrum. The presence of high Ig concentrations in the colostrum consumed by the neonate coincides with an extensive, but short-lived,

nonspecific macromolecular absorption by the neonate intestine. In contrast to ungulate species, the human fetus acquires systemic IgG primarily during the last trimester of gestation *via* transport across the placental membrane. Gut closure in the human infant occurs before birth, with little Ig being absorbed intact after birth (Brandtzaeg, 2003; Brandtzaeg and Johansen, 2007). A third group of species includes those in which the Ig is transferred both *via* placenta and mammary secretions (rodents and carnivores).

For rodents, carnivores, and ungulate species, consumption of adequate quality and quantity of colostrum is important for the offspring to provide systemic immune protection in the short term. In the case of human infants, colostrum consumption is more important for protection of the gastrointestinal tract, consistent with the lower total Ig content in human colostrum relative to other species, especially the lower IgG content (Table 9.1, Fig. 9.2). An additional consequence of the different routes of Ig transmission to the young relates to the changes in the relative contents of Ig that occur in the transition from colostrum to milk within certain species (illustrated in Fig. 9.2). Indeed, the distribution of Ig in human colostrum is similar to that in human milk, whereas the high concentration of IgG in colostrum of other species rapidly declines with successive milking or nursings, while the proportion of IgA increases between colostrum and milk for many species. These rapid changes in relative proportions of the Ig are characteristic of ungulate species and rodents where colostrum and milk Ig provide immune protection both systemically and for the gastrointestinal tract.

9.4.2 Intestinal Uptake of Immunoglobulins

Intestinal uptake of macromolecules, including Ig, occurs by an endocytic pathway in the calf and pig (Staley and Bush, 1985; Sangild *et al.*, 1999). For a period after birth, this pathway results in the transport of macromolecules across the enterocyte, followed by release into the *lamina propria* from which the macromolecules can be absorbed into the lymphatics or portal circulation. Intestinal closure occurs when this macromolecular transport is terminated even though uptake of macromolecules into enterocytes may continue (Staley and Bush, 1985). Transport of macromolecules occurs primarily in the small intestine and particularly in the jejunum (Staley and Bush, 1985). Selectivity of transport of macromolecules by the neonate intestine varies with species. In the newborn human, guinea pig, and rabbit, little Ig is transported across the enterocytes and the intestine is selective to the point of exclusion of all proteins. In contrast, ungulate species exhibit little selectivity toward proteins which are absorbed prior to closure. Rodents form an intermediate group in which there is high selectivity in the transport of IgG across the intestinal barrier which occurs *via* the FcRn (Rodewald and Kraehenbuhl, 1984; Simister and Mostov, 1989; Ahouse *et al.*, 1993). Selective transport of IgG by the rat intestine continues for about 3 weeks.

Intestinal closure generally is considered to be completed in ruminants by about 24 h after birth and in about 36 h in pigs and horses. Loss of absorptive capacity of the intestine begins soon after birth and progresses continuously until closure is complete. The process of closure is affected by environmental stress, by severe dystocia, and possibly by the nutritional status of the calf (discussed in Davis and Drackley, 1998). Failure of transfer of passive immunity results in significant risk of disease for the neonate. Failure of passive transfer is generally considered to have occurred when a calf's blood IgG concentration at 48 h after birth is less than 10 mg/mL (Bovine Alliance on Management and Nutrition, 1995). Failure of passive transfer resulting in a low serum Ig concentration in calves is often associated with increased calf mortality and disease, and with decreased growth (Nocek *et al.*, 1984; Donovan *et al.*, 1986; Robison *et al.*, 1988; Selim *et al.*, 1995; Wells *et al.*, 1996), and may be associated with decreased milk production when the calf matures (DeNise *et al.*, 1989). Maternal IgG in the calf's blood gradually declines over the initial month after birth, with a half-life of approximately 16 days (Husband *et al.*, 1972).

9.5 Immunoglobulin Function in the Neonate

9.5.1 Immunoglobulins and Immunity

The mammalian immune system is highly complex and robust, with many interacting components and significant functional redundancy. Innate immunity primarily consists of cells that mount rapid and nonspecific responses to pathogen exposure (Moser and Leo, 2010; Sun *et al*., 2011). This set of cells includes granulocytes, macrophages, and dendritic cells, which are relatively short-lived and which respond in an identical manner to pathogen reexposure as in their initial exposure. Activation of the innate immune response occurs *via* toll-like receptor-mediated and toll-like receptor-independent recognition of pathogens (Moser and Leo, 2010; Saiga *et al*., 2011). The toll-like receptors recognize a wide spectrum of pathogenic organisms (Moser and Leo, 2010). Dendritic cells also monitor pathogen exposure at mucosal surfaces and contribute to the mucosal immune system (Iwasaki, 2007). Adaptive or acquired immunity, on the other hand, generally is considered in terms of T and B lymphocytes, which respond to pathogen challenge more slowly but with high specificity (Moser and Leo, 2010; Liongue *et al.*, 2011; Sun *et al.*, 2011). The latter feature of the adaptive response occurs as a consequence of somatic rearrangement of genes generating highly diverse sets of antigen receptors. Clonal expansion of the antigen-specific lymphocytes leads to a population of long-lived memory cells, a hallmark of the adaptive immune system (Moser and Leo, 2010; Sun *et al.*, 2011). Interactions between the intestinal microbiota and the intestinal innate and adaptive immune components are essential for maintaining gut health (Jarchum and Pamer, 2011).

Immunoglobulins are produced as part of the adaptive immune responses (Butler and Kehrli, 2005; Moser and Leo, 2010). Antigen-specific Ig is produced and secreted by activated B lymphocytes in response to antigen exposure and released into the blood and body fluids as part of the humoral immune protection. In this manner, the immunoglobulins provide a widely dispersed means of antigen recognition by the immune system. Immunoglobulin bound to the antigen may neutralize the effects of bacterial toxins and inhibit the infectivity of viruses. Opsonization of pathogens is the process where the Fab portion of the Ig binds to surface antigens. This renders the pathogen more susceptible to phagocytosis as a result of the pathogen-Ig complex binding to Fc receptors on innate immune cells (Radaev and Sun, 2001). In addition, IgG isotype antibodies can activate complement, providing another means of cell lysis and killing of the pathogen. While generally low in milk (Targowski, 1983), components of the complement system are expressed in response to intramammary pathogen or lipopolysaccharide challenge (Rainard *et al.*, 2008; Danielsen *et al.*, 2010). Milk antibodies play an important role in immune protection of the mammary gland (Sordillo *et al.*, 1997).

Immunoglobulin G is the primary Ig transferred from mother to neonate, whether the transfer occurs *via* colostrum, as in ungulate species, or *via* transplacental transfer, as in humans. In either case, the blood-borne IgG, which is produced as a response of the maternal adaptive immune system, would be expected to offer immune protection to the neonate through the mechanisms indicated above, including their contribution to antigen recognition in the phagocytic process characteristic of innate immune cells.

9.5.2 Intestinal Actions of Colostrum and Milk Immunoglobulins

In addition to the absorption of Ig from the intestine to provide systemic Ig, especially IgG, the Ig found in colostrum and milk has protective effects within the intestine. The value of colostrum and milk Ig, particularly IgA, for protection of the gastrointestinal tract is well established (Rejnek *et al.*, 1968; Renegar and Small, 1999). For example, while milk sIgA is not absorbed by the human infant's intestinal mucosa, the presence of

sIgA in the lumen contributes a level of protection for the intestinal epithelial barrier (Brandtzaeg, 2003; Brandtzaeg and Johansen, 2007; Russell, 2007). Secretory IgA is the primary Ig responsible for immune protection of mucosal surfaces, including the intestine (Brandtzaeg and Johansen, 2007). Milk sIgA can bind bacteria, toxins, and other macromolecules, thereby limiting their ability to bind to intestinal cells and preventing them from being transported across the mucosa where they may cause a systemic immune response (Fernandez *et al.*, 2003; Hanson *et al.*, 2005; Davids *et al.*, 2006). Microbe binding by sIgA modulates bacterial colonization of the gastrointestinal tract which impacts the interaction of those microbes with the developing neonate intestinal immune system (Brandtzaeg, 2003; Hanson *et al.*, 2005; Brandtzaeg and Johansen, 2007). In addition, IgA inhibits proinflammatory responses to oral antigens, thereby having a major role in the immunosuppression and oral tolerance mechanisms in the intestine (Brandtzaeg and Johansen, 2007). Breast feeding of human infants promotes the development of the local intestinal immune response and production of IgA (Prentice, 1987; Koutras and Vigorita, 1989).

Intestinal uptake of IgG after closure can occur *via* the FcRn receptor (Brandtzaeg and Johansen, 2007). Transport of IgG across the human adult intestinal enterocyte by FcRn seems to be bidirectional, suggesting that IgG is involved in immune surveillance and defense of the mucosal lining (Israel *et al.*, 1997; Dickinson *et al.*, 1999; Rojas and Apodaca, 2002; Yoshida *et al.*, 2004). Intestinal FcRn may deliver IgG-antigen complexes to the *lamina propria* for immune processing, resulting in enhanced local mucosal immune response (Brandtzaeg and Johansen, 2007; Rojas and Apodaca, 2002). Alternatively, functionally intact IgG remaining in the lumen may bind antigens and contribute to the intestinal protection (Guarner and Malagelada, 2003). An IgG Fc binding protein associated with the intestinal mucus may block uptake of IgG-antigen complexes, allowing the complexes to be degraded in the lumen (Kobayashi *et al.*, 2002; Siccardi *et al.*, 2005).

9.5.3 Nutritional Value of Colostrum and Milk Immunoglobulin

The lactose and protein, particularly the casein, in colostrum and milk generally are highly digestible, with 97% or more of these macronutrients being digested in the young animal (Devillers *et al.*, 2004; Le Dividich *et al.*, 2005; Lin *et al.*, 2009). In contrast, Igs tend to be more resistant toward digestion and can be identified within the intestinal lining after colostrum ingestion (Danielsen *et al.*, 2010). Intestinal digestion of Ig is among the slowest of the whey proteins. Immunoglobulin G provides the smallest proportion of absorbed amino acids to the neonate compared with other major whey proteins (Yvon *et al.*, 1993). In vitro studies indicate that IgA may be more resistant to intestinal digestion in lambs than is IgG (Stelwagen *et al.*, 2009). Bovine IgG1 is more susceptible to pepsin hydrolysis than IgG2, while IgG2 is more susceptible to trypsin (de Rham and Isliker, 1977). Immunoglobulins may be further hydrolyzed by pancreatic enzymes, where chymotrypsin preferentially hydrolyzes IgM over IgG and trypsin preferentially digests bovine IgG1 over IgM (Brock *et al.*, 1977).

Even though a substantial amount of Ig molecules are absorbed intact in the ungulate neonate before closure, ~75% of the Ig is either digested and absorbed as amino acids or small peptides, or passed through the gastrointestinal tract and may become a substrate for bacterial fermentation in the intestine or may be excreted *via* feces. For example, absorption of intact Ig in neonatal pigs has been reported in the range 5–25% (Jensen *et al.*, 2001; Bikker *et al.*, 2010; Lin *et al.*, 2009) relative to the amount supplied *via* colostrum. By comparison, in the case of adult humans consuming a bovine whey protein concentrate, approximately 59 and 19% of ingested Ig is still detectable in effluents from the jejunum and ileum, respectively (Roos *et al.*, 1995). This compares with estimates of digestion of milk proteins in adult humans which are about 42 and 93% complete at the end of the jejunum and the ileum, respectively (Mahe *et al.*, 1992).

The efficiency of the absorption of Ig depends on the nutrients that are ingested along with the Ig, which suggests that colostrum composition may be a factor in determining Ig absorption (Bikker *et al.*, 2010). Immunoglobulin G absorption is greater when newborn piglets are fed porcine colostrum compared to bovine colostrum (Jensen *et al.*, 2001), perhaps relating to differences in colostrum composition between species which could affect the efficiency of Ig absorption.

9.5.4 Role of Colostrum

For the neonate, consumption of colostrum bridges the abrupt transition from a parenteral (*via* placenta) nutrient source to an enteral supply of nutrients (Siggers *et al.*, 2011). While the transfer of maternal immunity is critical for the neonate in order to reduce morbidity and mortality, it should also be stressed that colostrum serves other important purposes to ensure survival, development, and well-being of the neonate, including as a source of energy (Le Dividich *et al.*, 2007), growth factors, and antimicrobial components (Pakkanen and Aalto, 1997).

Newborn mammals are born with low energy depots, and during the first few days after birth, sufficient intake of energy from colostrum is of paramount importance to avoid hunger and neonatal death. For example, newborn piglets are born with low energy depots (limited glycogen stores in the liver and muscles), and these depots are sufficient for maintaining normal piglet behavior for only about 16 h after birth (Theil *et al.*, 2011). Piglets with low colostrum intake are at the risk of dying in the perinatal period. Significant variation exists among sows in colostrum yield, as well as colostrum intake among littermates (Farmer and Quesnel, 2009). Large birth weight, small litters, and low number in the birth order are factors that are associated with a high intake of colostrum (Le Dividich *et al.*, 2005; Farmer and Quesnel, 2009). Each of these factors will influence the amount of colostrum Igs consumed by the neonate.

Colostrum has a high content of growth factors such as insulin-like growth factor, epidermal growth factor, and transforming growth factors (Pakkanen and Aalto, 1997). Some growth factors act by stimulating proliferation of the small intestine and increase the villous height and the absorptive capacity (Blum, 2003), and extensive growth of the intestine occurs during the first days of life (Xu, 1996). However, if inadequate amounts of colostrum are ingested, the intestine of the newborn offspring will start to degenerate and induce gut dysfunction, which in turn will lead to bacterial overgrowth, inflammation, and subsequently excessive nutrient fermentation (Siggers *et al.*, 2011).

Colostrum and milk contain a range of antimicrobial factors and factors that may impact the immune system (Pakkanen and Aalto, 1997; Playford *et al.*, 2000; Hanson *et al.*, 2001, 2005; Barrington and Parish, 2001; Gill, 2003; Lonnerdal, 2003; Siccardi *et al.*, 2005; Blum, 2006; Newburg and Walker, 2007; Mehta and Petrova, 2010). In addition to Igs, these include the iron-binding antimicrobial protein, lactoferrin; the antibacterial enzyme, lactoperoxidase; the antibacterial and lytic enzyme, lysozyme; oligosaccharides that function as analogues of microbial ligands on mucosal surfaces; antimicrobial heat-stable peptides (defensins); and soluble CD14. Colostrum and milk also contain leukocytes, including activated neutrophils, macrophages, and lymphocytes. The relative concentrations of these factors vary considerably among species.

9.6 Manipulation of Mammary Gland Immunity

9.6.1 Enhancing Homologous Transfer of Immunity

Vaccination or natural immunization of cows, ewes, and sows against enterotoxigenic bacteria (Wilson *et al.*, 1972; Kortbeek-Jacobs *et al.*, 1984; Moon and Bunn, 1993) or intestinal viruses (Saif *et al.* 1984; Lanza *et al.*, 1995) can provide enhanced protection for the newborn and decrease

neonatal morbidity and mortality. Feeding of newborn calves with a pooled colostrum from cows immunized against bovine rotavirus can affect the incidence and duration of diarrhea observed in those calves (Parreño *et al.*, 2004, 2010). Immunization of sows against transmissible gastroenteritis virus enhances the protective value of the sow colostrum and milk for the piglet (Aynaud *et al.*, 1991; Salmon, 1995). Other examples provide evidence that antibodies passively transferred to the human infant from mothers who became naturally immunized against enteric pathogens can reduce the incidence of diarrhea in the infant (Glass *et al.*, 1983; Ruiz-Palacios *et al.*, 1990; Lilius and Marnila, 2001).

9.6.2 Heterologous Transfer of Immunity

There also is considerable promise in the potential for heterologous transfer of passive immunity *via* products derived from colostrum or milk (Levine, 1991; Facon *et al.*, 1993; Davidson, 1996; Mestecky and Russell, 1998; Weiner *et al.*, 1999; Zeitlin *et al.*, 2000; Korhonen *et al.*, 2000b; Zinkernagel, 2001; Uruakpa *et al.*, 2002; Gapper *et al.*, 2007; Struff and Sprotte, 2007, 2008; Stelwagen *et al.*, 2009; Hurley and Theil, 2011). For example, bovine colostral Ig preparations from immunized cows have been effective for disease protection of the neonate in swine (Cordle *et al.*, 1991; Schaller *et al.*, 1992) and animal models such as mice (Jenkins *et al.*, 1999; Huang *et al.*, 2008). Intragastric gavage of rabbit pups with human secretory IgA protects against challenge with *Escherichia coli* K100 (Maxson *et al.*, 1996).

The opportunity to use antigen-specific vaccination to manipulate the immunological status of animals and then harvest the resulting colostrum or milk as a potential means of enhancing human health has been recognized since at least the 1950s (Campbell and Petersen, 1963; Lascelles, 1963). Consumption of immune milk from cows inoculated against human respiratory diseases has been proposed as a means of slowing disease outbreaks before reaching epidemic levels (Alisky, 2009). Several immune milk products are available commercially (McFadden *et al.*, 1997; Uruakpa *et al.*, 2002; Struff and Sprotte, 2007, 2008; Stelwagen *et al.*, 2009). Safety of such bovine immune milk products for human use has been discussed by others (Bernhisel-Broadbent *et al.*, 1991; Colker *et al.*, 2002; Gingerich and McPhillips, 2005; Struff and Sprotte, 2008). Hyperimmunization of cows with the intent of harvesting immune colostrum or milk for human use has been demonstrated for human rotavirus, for several species of enteropathogenic bacteria, for enterotoxigenic *E. coli* strains associated with traveler's diarrhea and for those strains that cause diarrhea in AIDS patients, for bacteria associated with the formation of dental caries, for cryptosporidiosis, and for other diseases.

9.7 Conclusion

Immunoglobulins in colostrum and milk represent an important component of the life line that links the mother and her offspring. The repertoire of antibody specificity found in colostrum and milk represents a history of immunological response of the lactating mammal to her environment, which then is shared with the offspring. The mechanism by which these maternal antibodies are passed to the offspring to provide systemic immunity is coordinated with the relative Ig concentrations in colostrum. Similarly, the role of colostrum and milk Igs in immune protection in the gastrointestinal tract of the young aligns with the type of Ig present in the secretion. Considerable value has been gained from understanding these features of Ig transmission to colostrum and milk for the benefit of rearing the young. Furthermore, the ability to manipulate the immune system of the pregnant or lactating animal allows for the application of the resulting mammary secretions in the control or treatment of disease in humans and other species.

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Lactoferrin

10

B. Lönnerdal and Y. A. Suzuki

10.1 Introduction

Iron-binding proteins exert many physiological functions in biological systems. Several of these proteins are involved in the transport of iron within the body and its storage in various compartments, while at the same time protecting against the pro-oxidant effects of iron. Other iron-binding proteins are enzymes that require iron as a cofactor for their activity.

Although lactoferrin was isolated and characterized in the late 1950s, there is still limited evidence that this protein has a significant biological function(s) in vivo. It has been suggested that lactoferrin is involved in several physiological events, such as bacteriostatic/bactericidal effects, being a component of the immune system, a growth factor, and an enhancer of iron absorption. These possible biological functions have led to interest in commercial applications of lactoferrin, and purified bovine lactoferrin and human recombinant lactoferrin are now commercially available in large quantities. Lactoferrin has received attention as a nutritional additive in infant formulae, food supplements, and other health-benefit products. In addition, various clinical trials to evaluate the efficacy of either human recombinant or bovine lactoferrin have been conducted, showing feasibility of this multifunctional glycoprotein for pharmaceutical purposes in several diseases such as cancer, periodontal disorders, and wound healing.

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10.2 Biochemical Properties of Lactoferrin

10.2.1 History, Discovery, and Presence in Biological Fluids

Lactoferrin was first identified in bovine milk by Sørensen and Sørensen (1939) and subsequently isolated from human milk and characterized by Johansson (1960). This red-colored protein was soon recognized as an iron-binding protein with biochemical characteristics similar to, but not identical to, those of transferrin. (Lactoferrin has also been called "lactotransferrin" which is less correct as it is distinctly different from the transferrin family of proteins.) It was subsequently found to be present in most exocrine fluids, such as saliva, bile, pancreatic fluid, amniotic fluid, and tears. Blood plasma also contains lactoferrin, but at a concentration several orders of magnitude lower than that in milk (Scott, 1989). During inflammatory reactions, certain cell types (e.g., neutrophils) accumulate lactoferrin, most likely from the plasma pool (Slater and Fletcher, 1987).

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10.2.2 Concentrations and Species Differences

The concentration of lactoferrin in milk varies widely among species. Human milk and the milk of other primates, pigs and mice are rich in lactoferrin, while the milk of other species, e.g., the cow and other ruminants, are low in lactoferrin and that of others (e.g., the rat) completely lacks this protein. Species that have a low concentration of lactoferrin usually have a high level of transferrin in their milk, whereas species like the human have very little transferrin in their milk (Masson and Heremans, 1971). Thus, it appears that most species primarily secrete a transferrin- or a lactoferrin-like protein in the milk, with the exception of the mouse, which secretes both types of protein at significant concentrations in its milk.

Maternal iron intake or status does not appear to affect the concentration of lactoferrin in milk (Zavaleta *et al.*, 1995b), nor does maternal infection during early or established lactation (Zavaleta *et al.*, 1995a; Lönnerdal *et al.*, 1996).

10.2.3 Molecular Weight and Glycosylation

Lactoferrin is a single-chain protein with a molecular mass of around 80 kDa. The sequence has been determined by both amino acid (Metz-Boutigue *et al.*, 1984) and nucleotide (Rey *et al.*, 1990) sequencing. The protein contains intramolecular disulfide bonds but no free sulphydryl groups. Lactoferrin is glycosylated at two distinct sites; the N-linked glycans have been characterized both with regard to monosaccharide structure and conformation (Spik *et al.*, 1988). Human milk lactoferrin contains poly-*N*-acetyllactosaminic glycans and the glycans of lactoferrin isolated from polymorphonuclear leukocytes seem to have an identical structure. Recently, the carbohydrate chains from human lactoferrin have been shown to be responsible for Toll-like receptor 4 activation (Ando *et al.*, 2010). This is quite an intriguing feature and may disentangle the complicated mechanisms behind the immunomodulating effect of lactoferrin, which will be discussed in more detail later. Bovine lactoferrin is also glycosylated but is characterized by having α l→3-linked galactose residues at the terminal nonreducing position. Furthermore, it contains additional glycans of the oligomannosidic type (Spik *et al.*, 1988). Lactoferrin has a high isoelectric point, pH 8.7 (Baker and Lindley, 1993), and therefore has a tendency to associate with other molecules due to charge differences.

10.2.4 Tertiary Structure

The polypeptide chain of lactoferrin consists of two globular lobes (Fig. 10.1) which are linked by an extended α-helix which is sensitive to proteolytic attack (Anderson *et al.*, 1989). The two domains have a similar amino acid sequence which is believed to be the result of an early duplication of an ancestral gene. Each lobe contains one iron-binding site and one glycan. However, the conformations of the N-terminal lobe and the C-terminal lobe are different and their affinity for iron is slightly different also (Anderson *et al.*, 1989). In its iron-free "apoform" the conformation of the lobes changes and lactoferrin becomes a more "open" molecule, which may explain the difference in susceptibility to proteolytic enzymes (see below). The tertiary structures of bovine and human lactoferrin have been characterized at a resolution of 2.8 Å (Anderson *et al.*, 1989).

10.2.5 Metal- and Anion-Binding Properties

The most common metal ion associated with lactoferrin in vivo is iron in its ferric (iron III) form (Anderson *et al.*, 1989). However, it has been shown that lactoferrin can also bind other metal ions such as copper, chromium, manganese, and aluminum in biological systems (Ainscough *et al.*, 1979). The proportion of lactoferrin molecules occupied by these other cations may be quite small; lactoferrin isolated from human milk was found to contain 2,000 times more iron than manganese (Lönnerdal *et al.*, 1985). In vitro, it is possible to

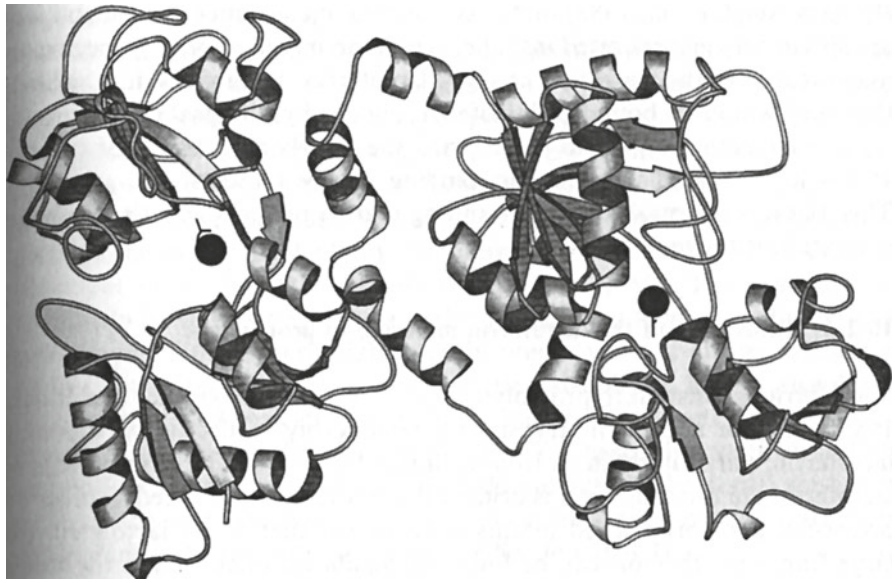

Fig. 10.1 Tertiary structure of bovine lactoferrin (picture courtesy of Drs. E.N. Baker and R. Kidd, University of Auckland)

find other metal ions (e.g., zinc) associated to lactoferrin under specific conditions, such as very low ionic strength (Blakeborough *et al.*, 1983), but it is questionable whether these metal ions are specifically incorporated into the lactoferrin molecule or nonspecifically associated with the negatively charged lactoferrin. Regardless, it is unlikely that they would be bound to lactoferrin under physiological conditions.

For each cation bound to lactoferrin, one bicarbonate or carbonate anion is incorporated into the iron-binding crevice (Anderson *et al.*, 1989). This anion is essential for metal binding, and its presence greatly facilitates saturation with iron.

10.2.6 Resistance of the Lactoferrin Molecule to Proteolysis

Lactoferrin is resistant to proteolytic degradation in vitro (Brines and Brock, 1983). Trypsin and chymotrypsin are remarkably ineffective at digesting lactoferrin, particularly in its iron-saturated form. Some large fragments of lactoferrin are formed, but it is evident that proteolysis is limited. Studies on breast-fed and formula-fed infants have shown that intact lactoferrin (or large fragments thereof) can be found in small but physiologically significant quantities in the stools (Spik *et al.*, 1982; Davidson and Lönnerdal, 1987). Thus, lactoferrin can survive digestion by pepsin and pancreatic enzymes in the infant gut to some extent and possibly exert biological functions in the gastrointestinal tract. In a study on cannulated pigs using ^{15}N-labeled proteins, it was shown that more lactoferrin than casein could be recovered intact from the ileum of suckling animals but not from adult pigs (Drescher *et al.*, 1999). Although only a minor proportion of the total milk lactoferrin may survive intact, this quantity is more than adequate to carry all the iron originally present in human milk (the potential role of lactoferrin in iron absorption will be discussed in Sect. 10.4.9). An even higher proportion of lactoferrin is found in the feces of premature infants (Donovan *et al.*, 1989); in addition, intact lactoferrin has been found in the urine of such infants, demonstrating that not only do some lactoferrin molecules survive digestion, but they may also be absorbed, circulated, and excreted in an intact form (Hutchens *et al.*, 1991).

In adults, ~60% of bovine apo-lactoferrin (20% iron-saturated) and ~80% of bovine holo-lactoferrin (hLf) has been shown to survive

in the stomach of healthy volunteers (Troost *et al.*, 2001), while recombinant hLf from transgenic cows was completely digested (Troost *et al.*, 2002). Thus, in human adults, bovine lactoferrin is more resistant to proteolytic degradation than human lactoferrin, making it conceivable that bovine lactoferrin will be active in the human adult gastrointestinal tract. It should be noted, however, that ingested lactoferrin was ~4.5–5 g, which is a very large dose, and may not reflect doses used in clinical trials.

10.3 Molecular Biology of Lactoferrin

10.3.1 The Lactoferrin Gene

The complete cDNA for human lactoferrin has been isolated from a mammary gland cDNA library and the amino acid sequence has been deduced from the nucleotide sequence (Rey *et al.*, 1990). The cDNA encodes a protein with a signal peptide of 19 amino acids followed by a mature protein of 691 residues. The cDNA and amino acid sequence of bovine lactoferrin have also been reported (Mead and Tweedie, 1990), as has the mRNA sequence (Goodman and Schanbacher, 1991). The mature protein consists of 689 amino acids and it has a 19 amino acid signal peptide. The nucleic acid sequence and the deduced amino acid sequence of the mature protein of bovine lactoferrin are homologous with published sequences for human lactoferrin (77% and 68%, respectively).

Regulation of lactoferrin synthesis is tissue-specific; expression of mammary gland lactoferrin has been shown to be dependent on prolactin in organ culture (Green and Pastewka, 1978) and unaffected by estradiol, whereas the synthesis of uterine lactoferrin is stimulated by 17-β-estradiol treatment in the immature mouse (Teng *et al.*, 1989). The expression of lactoferrin in the bovine mammary gland is different from that of other milk proteins in that it is very high in early pregnancy and during involution; during lactation it is low in actively secreting alveoli and high in alveoli that have accumulated vesicles in the lumen and secretory epithelium, which is indicative of stasis (Molenaar *et al.*, 1996). These observations and the finding that lactoferrin is expressed predominantly in the ductal epithelium close to the teat are consistent with an antibacterial role of lactoferrin, particularly with regard to mastitis (see Sect. 10.4.2).

10.3.2 Recombinant Human Lactoferrin

Recombinant human lactoferrin was first expressed in baby hamster kidney cells (Stowell *et al.*, 1991). The expressed protein was shown to be virtually identical to that isolated from human milk when migration patterns on SDS-polyacrylamide gels and the presence of glycan chains were compared. Interestingly, all the recombinant lactoferrins purified from the cell culture medium were in a fully iron-saturated form. It has subsequently been produced in *Saccharomyces* (Liang and Richardson, 1993), *Aspergillus nidulans* (Ward *et al.*, 1992), cows (Krimpenfort, 1993), baculovirus-insect cells (Salmon *et al.*, 1997), tobacco plants (Salmon *et al.*, 1998), and rice (Suzuki *et al.*, 2003). Large-scale production of human lactoferrin for clinical trials has been achieved in transgenic cows and *Aspergillus awamori* (Ward *et al.*, 1995) and in transgenic rice (Nandi *et al.*, 2002). The recombinant forms appear to have iron-binding properties identical to native lactoferrin, even after mild heat treatment (Mata *et al.*, 1998), ability to bind to cellular receptors and stability against proteolytic enzymes (Suzuki *et al.*, 2003), although the size and composition of the glycan appear to be somewhat different. The three-dimensional structure of the recombinant human lactoferrin expressed in *A. awamori* has been determined by X-ray crystallography. The main-chain atoms for the entire polypeptide can be superimposed and there are no significant differences in side-chain conformations or in the iron-binding sites (Sun *et al.*, 1999). Recombinant lactoferrins expressed in various expression systems maintain structural and functional properties virtually identical to native lactoferrin, suggesting that the structure of the protein is not affected by the mode of expression.

Iron from ferrous sulfate and from recombinant human lactoferrin produced in rice are equally well utilized in human adults (Lönnerdal and Bryant, 2006). Addition of recombinant human lactoferrin and lysozyme, both expressed in rice, into oral rehydration solution (ORS) has been shown to be beneficial for children with acute diarrhea and dehydration (Zavaleta *et al.*, 2007). In addition, recombinant human lactoferrins expressed in other sources are also under intensive investigation for clinical applications, especially for inhibition of carcinogenesis (Jonasch *et al.*, 2008; Hayes *et al.*, 2010) and for treating foot ulcers (Lyons *et al.*, 2007; Engelmayer *et al.*, 2008).

10.4 Biological Functions of Lactoferrin

Since lactoferrin was identified immediately as an iron-binding protein, it is logical that most biological functions for lactoferrin suggested initially were related to this property. Lactoferrin was found to bind specifically to intestinal biopsies and was proposed to be involved in the regulation of iron uptake by the mucosa. Because of its high concentration in the milk of some species, lactoferrin was also proposed to be involved in the delivery of iron into milk. The low degree of iron saturation of lactoferrin in human milk and its exceptionally high affinity constant for iron also prompted suggestions that lactoferrin is a bacteriostatic agent. This was supported by in vitro experiments; addition of iron to human milk or lactoferrin abolished the bacteriostatic effect. Since only some bacterial strains are affected by lactoferrin, it was suggested that the presence of lactoferrin in the diet could affect the fecal bacterial flora. Another possible function of lactoferrin is in macrophages, where high concentrations of lactoferrin accumulate from activated neutrophils during inflammation and therefore may help with phagocytic killing.

While some of the proposed biological functions for lactoferrin still hinge on its iron-binding capacity, other suggested functions appear to be unrelated to iron. For example, lactoferrin has been shown in some test systems to have a growth-stimulatory effect. This effect was seen for both iron-saturated lactoferrin and apolactoferrin in a study during which attempts were made to assure that these states were not changed during the experiment (Nichols *et al.*, 1990). Further, the described bactericidal effect of lactoferrin has been identified as a region of the molecule that is not involved in iron binding (Bellamy *et al.*, 1992). Several other proposed functions of lactoferrin in the immune system may not be dependent on the iron saturation of lactoferrin. Lactoferrin has also been shown to be involved in a wide variety of biological functions (as described in more detail below) and the multifunctionality of lactoferrin is now generally accepted. A graphic depiction of the multifunctionality of lactoferrin is shown in Fig. 10.2.

10.4.1 Lactoferrin and Immune Function

During an inflammatory response, lactoferrin is released into circulation by activated neutrophils and it has been proposed that this increased level of circulating lactoferrin is partially responsible for "hyposideraemia of inflammation" by removal of iron from transferrin and incorporation into the reticuloendothelial system (Van Snick and Masson, 1976). However, it is not known whether the rate of iron transfer from transferrin to lactoferrin is sufficiently high at physiological pH in order to mediate hyposideraemia. In addition, mice exhibited IL-l-induced hyposideremia, even in the presence of neutropenia (a deficiency of granulocytes). Although these findings indicate that lactoferrin may be unimportant for iron scavenging during inflammation, lactoferrin in synovial fluid of rheumatoid arthritis patients can prevent iron-mediated tissue damage by reducing free synovial iron (Guillen *et al.*, 2000). In addition, a biological importance of lactoferrin in host defense is emphasized by the observed susceptibility of subjects with congenital or acquired lactoferrin deficiency to recurrent infections (Boxer *et al.*, 1982).

It has been suggested that lactoferrin plays a regulatory role during cytokine responses (Machnicki *et al.*, 1993). At concentrations lower

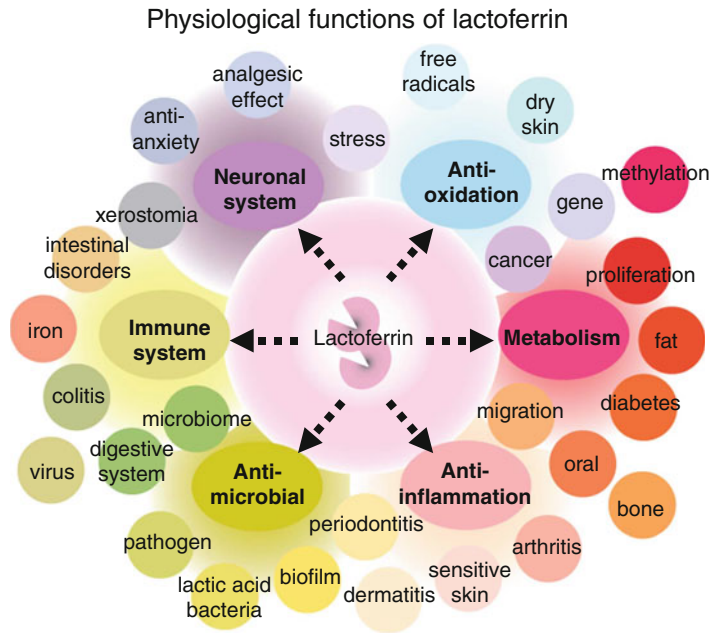

Fig. 10.2 Multi-functionality of lactoferrin

than 10^{-8} M, it has been reported that lactoferrin is an inhibitor of cytokine responses in vitro, suppressing the release of IL-1, IL-2, and tumor necrosis factor (TNF) from mixed lymphocyte cultures (Crouch *et al.*, 1992). The biological action of TNF, IL-1, or IL-2 is not blocked, therefore suggesting a regulatory role. IL-6, IL-10, and nitric oxide are all down-regulated by human lactoferrin in mononuclear cells in vitro and in vivo, in response to lipopolysaccharide (LPS) activation (Kruzel *et al.*, 2002). The down-regulation of IL-6 secretion induced by TNF-α resulted from the inhibition of NF-κB binding to the TNF-α promoter. On the other hand, lactoferrin stimulates the production of colony-stimulating factor both in vitro and in vivo (Sawatzki and Rich, 1989). Proinflammatory interferon (IFN) γ and TNF-α in transgenic mice carrying a functional human lactoferrin gene was stimulated to higher levels by *Staphylococcus aureus* compared with congenic controls (Guillen et al., 2002).

The other mechanism responsible for the anti-inflammatory activity is based on the interaction between human lactoferrin and macrophages through CD14. CD14 forms a complex with LPS, and the complex induces IL-8 secretion from endothelial cells. This IL-8 secretion is inhibited by lactoferrin in endothelial cells (Elass *et al.*, 2002). This mechanism to protect animals from septic shock induced by LPS has been confirmed in vivo (Baveye *et al.*, 2000).

Topical application of lactoferrin has an anti-inflammatory effect at the sites of skin inflammation where TNF-α is an important mediator, which facilitates a migration of epidermal Langerhans cells, resulting in an inflammatory response. Lactoferrin inhibits the action of IL-1β, which otherwise mediates TNF-α production (Griffiths *et al.*, 2001; Kimber *et al.*, 2002; Cumberbatch *et al.*, 2003; Kruzel *et al.*, 2006). Lactoferrin also decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma (Kruzel *et al.*, 2006; Chodaczek *et al.*, 2007). A novel anti-inflammatory property of lactoferrin has lately been reported in which lactoferrin inhibits migration of granulocytes by regulating cell adhesion and motility through granulocyte signaling pathways (Bournazou *et al.*, 2009).

Oral administration of bovine lactoferrin may also modulate the intestinal immune system. Lactoferrin strongly up-regulates IL-18 at the small intestinal epithelium, which then stimulates IFN-γ and activates T and NK cells, and therefore exhibits anticancer activity (Iigo *et al.*, 2004).

A recent report introduced a novel feature including the function of glycans in human lactoferrin (Ando *et al.*, 2010). Human lactoferrin was found to induce moderate activation of Toll-like receptor 4 (TLR4)-mediated innate immunity through its carbohydrate chains. TLR4 is known to trigger both myeloid differentiating factor 88 (MyD88)-dependent and MyD88-independent signaling pathways, and human lactoferrin activated both pathways. Tumor necrosis factor receptor-associated factor 6 (TRAF6), which is indispensable in MyD88-dependent pathways, is necessary for the NF-κB activation by human lactoferrin, but TRAF2 and TRAF5 are not required. On the other hand, LPS-dependent TLR4 activation was suppressed by human lactoferrin but not by the carbohydrate chains of human lactoferrin, indicating that its polypeptide moiety is responsible for this reaction.

10.4.2 Bacteriostasis/Bactericidal Effects

Due to the iron-sequestering properties of lactoferrin, it was hypothesized that the presence of lactoferrin would impede iron utilization by bacteria and result in bacteriostasis. Bovine lactoferrin in the apo-form has been shown to have bacteriostatic activity against mastitic *Escherichia coli* (Rainard, 1986). However, a few strains were resistant or unaffected, indicating that mechanisms other than simple iron withholding may be involved in the antimicrobial action of lactoferrin. Several mechanisms have been proposed. Lactoferrin has been shown to cause the release of LPS from the cell wall of Gram-negative bacteria (Ellison *et al.*, 1988). It was subsequently shown that lactoferrin and lysozyme have a synergistic effect on bacterial killing as the "pores" formed by the removal of LPS expose the inner membrane proteoglycan to lysozyme activity (Ellison and Giehl, 1991). A similar antibacterial effect of lactoferrin and lysozyme has been shown against Gram-positive *Staphylococcus epidermis*; in this case, lactoferrin binds to lipoteichoic acid (Leitch and Willcox, 1999). Erdei *et al.* (1994) showed that lactoferrin binds to porins, a group of molecules common in *E. coli*, thus causing changes in permeability. Infants fed human milk are known to be more resistant to intestinal infections than those fed formula, presumably due to the presence of considerable amounts of lactoferrin. Bacteriostatic effects of lactoferrin and human milk were demonstrated, and the effects could be abolished by addition of iron (Bullen *et al.*, 1972). Lactoferrin has also been shown to have weak ribonuclease activity and it has been suggested that this may assist in killing bacteria (Ye *et al.*, 2000).

It is known that the gut microflora of breast-fed infants is different from that of formula-fed infants; the former is composed of predominantly bifidobacteria, lactobacilli, and staphylococci, while the latter contains enterococci, coliforms, and bacteroides (Balmer *et al.*, 1989). Lactoferrin has been shown to promote the growth of *Bifidobacterium* spp. in vitro (Petschow *et al.*, 1999). However, supplementation of infant formula with bovine lactoferrin did not influence gut microflora markedly (Roberts *et al.*, 1992), indicating that lactoferrin may be acting in conjunction with other factors in breast milk, e.g., secretory IgA, lysozyme, citrate, and bicarbonate.

A domain of bovine and human lactoferrin, called lactoferricin, which is released by treatment with proteolytic enzymes, has been isolated and found to have bactericidal activity (Bellamy *et al.*, 1992). This peptide showed a marked growth inhibitory effect on *E. coli* O-lll (Saito *et al.*, 1991) and enterohemorrhagic *E. coli* 0157:H7 (Shin *et al.*, 1998). Several other antibacterial peptides of bovine lactoferrin have subsequently been isolated, and some have activity against *Listeria monocytogenes* (Dionysius and Milne, 1997). All these peptides are from regions that do not contain an iron-binding site. While the peptides were produced first in vitro, it was recently shown by affinity capture time-of-flight mass spectrometry that they are also formed from ingested lactoferrin in the human stomach (Kuwata *et al.*, 1998).

Lactoferrampin (residues 265–284), another cationic peptide, has recently been shown to have strong antimicrobial activity. This peptide originally exhibited candidacidal activity which was substantially higher than the activity of lactoferrin and was active against *Bacillus subtilis*, *E. coli*,

and *Pseudomonas aeruginosa* (van der Kraan *et al.*, 2004). The bactericidal activity was found to be much stronger in a chimera consisting of lactoferricin and lactoferrampin than in the constituent peptides (Bolscher *et al.*, 2009). Further, the negatively charged model membranes interacted with this chimera stronger than it did with either lactoferricin or lactoferrampin, suggesting that chimerization of the two antimicrobial peptides synergistically improves their biological activity. The effect of a fusion between lactoferricin and lactoferrampin was tested as an alternative to antimicrobial growth promoters in pig reproduction, and growth performance in piglets were significantly enhanced by supplementation with this lactoferricin-lactoferrampin fusion peptide (Tang *et al.*, 2009). Another interesting aspect of lactoferrin is that its N-terminal lobe possesses a serine protease-like activity (Qiu *et al.*, 1998), enabling it to cleave proteins in arginine-rich regions, and the protease active site is situated in the N-terminal lobe (Hendrixson *et al.*, 2003). Lactoferrin is capable of degrading some virulence proteins, key components for bacterial invasion which normally form a complex in the host cell membrane (Gomez *et al.*, 2003). Therefore, this degradation inhibits bacterial uptake into host cells. Lactoferrin also efficiently inhibits biofilm formation, especially that by *P. aeruginosa* (Singh *et al.*, 2002). Additionally, a study on the effect of lactoferrin on oral bacterial attachment (Arslan *et al.*, 2009) has revealed that initial attachment of *Streptococcus gordonii* was suppressed by lactoferrin. The antifungal activity of lactoferrin and lactoferricin has been tested mainly against *Candida*, with direct action on *Candida* cell membranes (Wakabayashi *et al.*, 1996). The antifungal activity of lactoferrin was not much higher than the commercially available antifungal drugs, but the combination of lactoferrin with the drugs has been shown to have additive or synergistic activity (Kuipers *et al.*, 1999).

10.4.3 Anticancer Effects

The effects of bovine lactoferrin on carcinogenesis have been investigated intensively. Orally ingested bovine lactoferrin is known to reduce the incidence and number of carcinomas in the colon (Sekine *et al.*, 1997), esophagus, lung (Ushida *et al.*, 1999), tongue (Tanaka *et al.*, 2000), and bladder (Masuda *et al.*, 2000). One suggested mechanism behind this effect is initiated by induction of cytokines such as IFN-γ and IL-18, which then activate T and NK cells (Wang *et al.*, 2000; Tsuda *et al.*, 2002). In IFN-γ knockout mice, consumption of bovine lactoferrin did not activate the IFN-γ/caspase-1/IL-18 effector pathway, but it was able to inhibit tumor growth and metastasis by activating an IFN-α/IL-7 effector pathway (Iigo *et al.*, 2009), suggesting the capability to activate multiple effector pathways. Some other possible factors associated with the anticancer effect of lactoferrin exist as well, such as down-regulation of a phase I detoxifying enzyme, cytochrome P450 1A2 (Fujita *et al.*, 2002), up-regulation of a phase II detoxifying enzyme, and glutathione-*S*-transferase, with consequent reduction in carcinogen activation (Tanaka *et al.*, 2000). Lactoferrin can also obstruct the transition from G1 to S phase (Damiens *et al.*, 1999) and from G0 to G1 phase (Xiao *et al.*, 2004) in the cell cycle of malignant cells. In addition, lactoferrin can promote apoptosis and arrest tumor growth in vitro. Bovine lactoferrin was seen to bring about an increase in the number of a death-inducing receptor, Fas, and a pro-apoptotic Bcl-2 family member, Bid, as well as in the activity level of caspase-8 and caspase-3 in the colon of tumor-bearing rats, which also explains, at least partially, the anticancer mechanism that lactoferrin possesses (Fujita *et al.*, 2004a, b). These apoptotic effects will likely be mediated by the immunomodulatory effect of lactoferrin.

Oral ingestion of recombinant human lactoferrin has also been shown to stimulate the same IL-18/IFN-γ effector pathway to exert anticancer activity (Varadhachary *et al.*, 2004). Furthermore, bovine apo-lactoferrin inhibits vascular endothelial cell tube formation (Shimamura *et al.*, 2004), and vascular endothelial growth factor (VEGF) mediates angiogenesis (Norrby *et al.*, 2001) in vitro, possibly leading to the suppression of tumor growth. However, it should also be noted that human apo-lactoferrin enhanced VEGF-

mediated angiogenesis (Norrby, 2004), indicating that species specificity must be considered for clinical applications of lactoferrin.

10.4.4 Antiviral Effects

Several studies suggest that lactoferrin has antiviral activity. Replication of HIV and human cytomegalovirus (CMV) were found to be inhibited by bovine or human lactoferrin in vitro (Harmsen *et al.*, 1995). However, the inhibition occurred at the stage of virus adsorption and/or penetration, and thus cell-bound viruses may be protected. Puddu *et al.* (1998) showed that both the apo- and holo-forms of bovine lactoferrin inhibit HIV replication in human T cells and also suggested that the antiviral activity is manifested at the early HIV-cell interaction. Lactoferrin has also been shown to inhibit the growth of respiratory syncytial virus (RSV) in vitro (Grover *et al.*, 1997) and to prevent rotavirus infection in human enterocyte-like cells in culture (Superti *et al.*, 1997). Enveloped viruses were susceptible to inhibition by lactoferrin either due to inhibition of the virus-host interaction exemplified by hepatitis B virus (Hara *et al.*, 2002), herpes simplex virus (Andersen *et al.*, 2004), and CMV (Hasegawa *et al.*, 1994) or direct interaction between lactoferrin and viral particles such as feline herpes virus (Beaumont *et al.*, 2003), hepatitis C virus (HCV) (Hara *et al.*, 2002), and HIV (Berkhout *et al.*, 2002). Naked viruses including rotavirus (Superti *et al.*, 2001), adenovirus (Arnold *et al.*, 2002), and enterovirus (Lin *et al.*, 2002) were also susceptible to inhibition by lactoferrin. Lactoferrin interacts with a variety of host cell surface molecules including heparan sulfate (Andersen *et al.*, 2004), which is likely responsible for efficient blocking of viral entry to the host cells.

Direct interactions of lactoferrin with various viruses have also been investigated in detail. Two envelope proteins, E1 and E2, in HCV have been shown to interact with both human and bovine lactoferrin (Yi *et al.*, 1997). The carboxyl region of lactoferrin (33 amino acid residues corresponding to amino acids 600–632) has been found to be the minimum binding site for the E2 protein and to prevent HCV infection in cultured human hepatocytes (Nozaki *et al.*, 2003). The envelope protein gp120 in HIV has been shown to interact directly and strongly with lactoferrin (Swart *et al.*, 1996). This interaction could shield the virus and inhibit virus fusion and entry into host cells.

Several clinical trials have been performed on patients with chronic hepatitis C (CHC) to clarify the effects of long-term oral administration of bovine lactoferrin. Oral administration of bovine lactoferrin (600 mg/day) to CHC patients (36 patients in the bovine lactoferrin group and 27 patients in the control group) for up to 3 months produced a Th1-cytokine-dominant environment in peripheral blood which favors the eradication of HCV by interferon therapy (Ishii *et al.*, 2003). Another group investigated the effect of combination therapy using consensus interferon (CIFN) and lactoferrin in CHC patients (18 patients in total) by a randomized controlled trial, and the combination therapy did not show any positive effect on virologic response (Hirashima *et al.*, 2004). Another randomized placebo-controlled trial investigated the combination of interferon plus ribavirin with oral lactoferrin for CHC patients (18 patients in each group), but it also failed to demonstrate any positive effects of lactoferrin after 24 weeks of treatment (Ishibashi *et al.*, 2005), whereas a similar treatment of a total of 111 CHC patients (50 patients with lactoferrin) concluded that lactoferrin is a potential useful adjunct treatment for CHC patients, based on a significant decrease in mean HCV RNA titer (Kaito *et al.*, 2007). In another randomized, double-blind, placebo-controlled trial, a megadose of bovine lactoferrin (1.8 g daily for 12 weeks) showed no significant effect, although the treatment was well tolerated and no serious toxicity was observed (Ueno *et al.*, 2006).

While many in vitro studies exhibit promising effects of lactoferrin towards HCV, most trials on CHC patients have failed to show any positive effect of lactoferrin, which indicates that oral administration may abrogate the active site of the lactoferrin molecule during digestion and absorption.

10.4.5 Lactoferrin as a Growth Factor

Milk, and particularly colostrum, has been shown to stimulate the proliferation of the small intestine (Berseth *et al.*, 1983; Heird *et al.*, 1984). Lactoferrin, being a major whey protein in the milk of some mammals, was suggested as a possible growth factor for the intestinal mucosa when Nichols *et al*. (1990) reported that thymidine incorporation into DNA of rat crypt cells was enhanced in the presence of human lactoferrin. This stimulation does not appear to be dependent on the presence of bound iron in human lactoferrin. The majority of lactoferrin in human milk is present in the iron-unsaturated form, indirectly supporting the above theory. A more distal anabolic effect was suggested in a study in which bovine lactoferrin orally administered to suckling pigs was found to stimulate protein synthesis in the liver (Burrin *et al.*, 1996).

Various cell lines have also been used to study the effects of lactoferrin on growth. Amouric *et al.* (1984) reported, based on their studies with a human enterocyte-like cell line (HT-29) in serum-free medium, that lactoferrin could not substitute for transferrin and was unable to support cell proliferation. In contrast, Oguchi *et al*. (1995) showed that iron-saturated bovine and human lactoferrin, as well as human transferrin, enhanced cell proliferation, whereas the iron-unsaturated forms suppressed it. In studies on MAC-T bovine mammary epithelial cells, lactoferrin has been shown to inhibit growth (Rejman *et al.*, 1992). However, one must note that the above studies primarily considered growth as a parameter and only one study examined differentiation which may be more significant when studying cells of intestinal origin. Clearly, further studies are needed to separate the two phenomena and to define the role/effects of lactoferrin on each of them. Our recent study revealed that only iron-free lactoferrin but not iron-saturated lactoferrin stimulates proliferation of human enterocyte Caco-2 cells though both forms of lactoferrin were internalized *via* clathrin-mediated endocytosis to the same extent (Jiang *et al.*, 2011). Interestingly, iron-free lactoferrin stimulated the extracellular signal-regulated mitogen-activated protein kinase (ERK) cascade to a greater extent than iron-saturated lactoferrin. The possibility of synergism should also be explored; one study showed enhanced cell proliferation and DNA synthesis in rat intestinal cells (IEC-6) in culture when EGF and lactoferrin were present together than the combined effect of each component given alone (Hagiwara *et al.*, 1995).

10.4.6 Effect on Bone Homeostasis

It has been shown that lactoferrin can accelerate bone formation by stimulating the proliferation and differentiation of osteoblasts and by inhibiting cell death (Cornish, 2004; Cornish *et al.*, 2004; Naot *et al.*, 2005). It also enhances the ability of osteoblasts to synthesize and mineralize bone matrix. These anabolic actions of lactoferrin in skeletal tissue are mediated by specific receptors, which are low-density lipoprotein receptor-related protein (LRP)-1 and -2 (Naot *et al.*, 2005).

Ovariectomized mice are often used as a postmenopausal animal model, and using this model, there are several studies assessing the effect of dietary lactoferrin on bone metabolism in vivo. The first study (Blais *et al.*, 2009) revealed that 27 weeks of supplementation with bovine lactoferrin improved bone mineral density and the femoral failure load in a dose-dependent manner. Another study revealed that lactoferrin dose-dependently improved bone formation and reduced bone resorption in response to suppression of serum TNF-α and IL-6 production and to elevation of serum calcitonin (Guo *et al.*, 2009). Osteoporosis is a major health issue among postmenopausal women. The effect of a lactoferrin supplement on bone health of postmenopausal women was examined based on the idea that lactoferrin could stimulate bone formation in osteoblasts. Because decreased angiogenesis may cause an imbalance of bone resorption and bone formation, ribonuclease, which may promote angiogenesis, was enriched in a lactoferrin supplement. After 6 months of treatment, ribonuclease-enriched lactoferrin significantly reduced

bone resorption and increased osteoblastic bone formation, suggesting that this treatment restores the balance of bone turnover in patients with osteoporosis (Bharadwaj *et al.*, 2009).

10.4.7 Effect on Wound Healing

Several studies have reported an effect of lactoferrin on wound healing both in vitro and in vivo. Treatment with bovine lactoferrin prior to UVB radiation effectively prevented damage to the corneal epithelium in rats (Fujihara *et al.*, 2000). Bovine lactoferrin also facilitated healing of human corneal epithelial wounds in vitro and enhanced platelet-derived growth factor (180-fold) and IL-6 (tenfold) responses (Pattamatta *et al.*, 2009).

Lactoferrin may be able to ameliorate chronic wounds such as diabetic foot ulcers, venous leg ulcers, and pressure ulcers because lactoferrin inhibits the formation of bacterial biofilms, which has been recognized as a major contributor to delayed wound closure. Lactoferrin and xylitol have been shown to disrupt synergistically the structure of the *P. aeruginosa* biofilm, which resulted in a significant reduction of bacterial viability. In situ analysis revealed that xylitol disrupted the biofilm structure and that lactoferrin permeabilized bacterial membranes (Ammons *et al.*, 2009). Topical application of recombinant human lactoferrin to diabetic neuropathic ulcers appeared to be safe and well tolerated and improved healing of ulcerative wounds (Lyons *et al.*, 2007).

Fibroblast and keratinocyte migration are also important during the process of wound healing. Lactoferrin has been shown to promote migration of fibroblasts in a wound-healing assay (Takayama and Mizumachi, 2001). Matrix metalloproteinase (MMP) regulates promotion of cell migration and MMP1 is activated by lactoferrin in fibroblasts (Oh *et al.*, 2001), suggesting that MMP1 up-regulation may be responsible for fibroblast migration.

10.4.8 Anti-adipogenic Effects

Anti-adipogenic effects have been reported recently as a novel function of lactoferrin. Lactoferrin has been shown to suppress adipogenic differentiation in human hepatocarcinoma (HepG2) and 3T3-L1 cell lines, and the number of lipid droplets decreased dose-dependently, suggesting a possible application of lactoferrin to control lipid metabolism (Yagi *et al.*, 2008). More recently, circulating lactoferrin levels were inversely associated with changes in levels of free fatty acids after fat overload (Fernandez-Real *et al.*, 2010), suggesting an important role of lactoferrin in fat metabolism through its anti-adipogenic activity as well as antioxidative and anti-inflammatory activities. A human study using a double-blind, placebo-controlled design with Japanese men and women has been conducted. Subjects consumed enteric-coated lactoferrin (300 mg/day as bovine lactoferrin) or placebo tablets for 8 weeks. X-ray computed tomography (CT) scanning images revealed that visceral fat area and subcutaneous fat area were significantly reduced in the lactoferrin group. Body weight, BMI, and hip circumference in the lactoferrin group also decreased significantly more than in the placebo group. This study suggests that lactoferrin is a promising agent for the control of visceral fat accumulation (Ono *et al.*, 2010). Trypsin-treated lactoferrin continued to show anti-adipogenic activity, but pepsin-treated lactoferrin had lost this activity (Ono *et al.*, 2011). Thus, for maintaining anti-adipogenic effects of lactoferrin when administered orally, enteric coating appeared to be necessary.

10.4.9 Lactoferrin and Iron Absorption

10.4.9.1 Clinical Studies

The hypothesis that lactoferrin is involved in the absorption of iron from breast milk was supported early by two observations. First, breast milk contains an unusually high concentration of lactoferrin and a major proportion of the iron in human milk is bound to lactoferrin (Fransson and Lönnerdal, 1980). Second, in spite of a relatively low concentration of iron in human milk, exclusively breast-fed infants maintain adequate iron stores up to at least 6 months of age (Siimes *et al.*,

1984; Lönnerdal and Hernell, 1994), suggesting a high bioavailability of breast milk iron. Radioisotope experiments on infants showed that iron absorption is higher from breast milk than from infant formula (Saarinen *et al.*, 1977). Indirect support for a higher bioavailability of iron from human milk than from formula has been obtained by several studies showing lower iron status of infants fed formula which had not been fortified with iron as compared to breast-fed infants (Saarinen and Siimes, 1977), although the concentration of iron in such formula was higher than in breast milk. Evidence that lactoferrin is the factor in breast milk responsible for this higher bioavailability is still inadequate.

Studies on nonhuman primate models (infant rhesus monkeys) have failed to demonstrate a pronounced positive effect of human or bovine lactoferrin on iron absorption (Davidson *et al.*, 1990). The infant rhesus monkey is considered to be an excellent model for the human infant as their gastrointestinal physiology is similar; monkey milk contains a high concentration of lactoferrin (Davidson and Lönnerdal, 1986) and it can be reared on regular infant formula without a need for adaptations in nutrient or energy content. In this study, iron absorption was relatively high from both infant formula and breast milk, possibly explaining why no further increase was observed. It is possible that recent modifications in the composition of infant formula, including the use of high levels of ascorbic acid, have optimized iron absorption. It is also possible that neither bovine nor human lactoferrin could play the same role as the species-specific monkey lactoferrin, even if their characteristics are similar. Studies on other animal models (mouse, rat) suggest a positive effect of lactoferrin on iron absorption/status (Fransson *et al.*, 1983; Kawakami *et al.*, 1988), although the validity of these models may be questionable.

In a study on iron absorption in full-term human infants using two stable isotopes of iron and a crossover design, breast-fed infants were fed either human milk or human milk from which lactoferrin had been removed specifically (Davidsson *et al.*, 1994). Iron absorption, estimated from erythrocyte iron incorporation, was slightly higher from the lactoferrin-free human milk than from intact human milk. This would argue against lactoferrin promoting iron absorption from breast milk and perhaps support an earlier hypothesis that lactoferrin inhibits the absorption of iron at an age when a need for iron is questionable (Brock, 1985). However, the age of the infant may be an important factor to consider when evaluating the involvement of lactoferrin in iron absorption. Most infants in the study were 4 months or older, as a certain quantity of stable isotopes was needed to allow detection of differences in iron incorporation. At this age, digestion has become much more efficient than at a younger age and, in fact, very small quantities of lactoferrin are found in the stools (Davidson and Lönnerdal, 1987). Although it is impossible to reach any conclusions based on only one infant, it is noteworthy that iron absorption was considerably higher from lactoferrin-containing breast milk than from lactoferrin-free milk in the only infant less than 3 months of age. It is obvious that further studies are needed to evaluate the effect of human lactoferrin on iron absorption in infants. Such studies may be facilitated by the availability of recombinant human lactoferrin.

The effect of bovine lactoferrin on iron absorption has also been evaluated in human infants. Results to date do not support a role for this protein in the absorption of iron by formula-fed infants. Three studies showed no significant difference in the iron status of infants fed formula supplemented with bovine lactoferrin compared to ferrous sulfate (Fairweather-Tait *et al.*, 1987; Chierici *et al.*, 1992; Lönnerdal and Hernell, 1994). In one study, iron status was marginally better in infants fed a high level of bovine lactoferrin as compared to a lower level of bovine lactoferrin or ferrous sulfate (Schulz-Lell *et al.*, 1991). However, it is not possible to draw any conclusion about the role of lactoferrin from this observation as the level of iron also was higher in the formula containing a higher level of lactoferrin. A recent randomized, placebo-controlled, double-blind study revealed that the hematocrit levels in bovine lactoferrin-supplemented infants

were significantly higher than those in the control infants, suggesting a potential beneficial effect on iron status by bovine lactoferrin (King *et al.*, 2007).

10.4.9.2 Studies on Cells and Biological Membranes

Specific binding of human lactoferrin to duodenal biopsies from adults was demonstrated by Cox *et al*. (1979). This finding suggested that lactoferrin may bind to certain sites in the small intestine and therefore be directly or indirectly involved in the acquisition of iron by the enterocyte. Studies on lactoferrin binding to brush border membrane preparations from mouse (Hu *et al.*, 1990), piglets (Gislason *et al.*, 1993), rhesus monkey (Davidson and Lönnerdal, 1988), and human infants (Kawakami and Lönnerdal, 1991) supported this hypothesis. It has also been shown in two human cell lines, HT-29 and Caco-2, that human lactoferrin binds to the cells in a saturable and specific manner (Mikogami *et al.*, 1994). These cell lines are colon carcinoma cells that, in culture, differentiate spontaneously into small intestinal cells with features characteristic of the enterocyte, including a brush border membrane. Both cell lines have been used in numerous studies on nutrient metabolism and are believed to be good models of the human small intestinal epithelial cell. Thus, lactoferrin has been documented to bind specifically to intestinal cells and the brush border membrane.

10.4.9.3 Uptake and Intracellular Processing of Lactoferrin and Iron

Dual isotope studies on human intestinal cells in culture have shown that both lactoferrin and iron are taken up by enterocytes (Mikogami *et al.*, 1994). These studies show, when monolayers are used to follow vectorial transport, that only a very small proportion of lactoferrin is transferred to the serosal side. Iron is therefore released within the cell and is rapidly complexed to another protein, possibly ferritin. Thus, lactoferrin is responsible for bringing iron into the intestinal cell, but the further fate of the iron is determined by other factors, such as the individual's need for iron. When iron status is low, it is likely that the internalized iron will be mobilized and transferred into the body, while in situations of satisfactory iron status, this iron may be lost in desquamated cells.

10.5 Lactoferrin Receptors

10.5.1 Lactoferrin Receptors in the Small Intestine

Lactoferrin receptors in the small intestinal mucosa were first reported by Mazurier *et al.* (1985) in rabbit brush-border membranes by ligand blotting. This followed the finding that human lactoferrin had the ability to deliver iron to mucosal cells of small intestinal biopsy tissues (Cox *et al.*, 1979), while bovine lactoferrin, human transferrin, and chick ovotransferrin did not. Studies on infant rhesus monkeys showed that rhesus lactoferrin and human lactoferrin bound to a receptor in the rhesus brush-border membrane in a specific and saturable manner (Davidson and Lönnerdal, 1988), whereas bovine lactoferrin and human transferrin showed no binding. The binding affinity of iron-saturated lactoferrin for the receptor was higher than that of apo-lactoferrin (Davidson and Lönnerdal, 1989). Studies on piglets (Gislason *et al.*, 1993) have documented a specific receptor on the brush border membrane with a K_d of 3×10^{-6} M and it was shown to be present in all segments of the small intestine. Human lactoferrin, bovine lactoferrin, and pig transferrin did not bind to the receptor. This degree of species specificity is noteworthy because porcine milk is known to contain lactoferrin as an iron carrier, and rat pup intestine has been reported to contain lactoferrin receptors, but no lactoferrin receptors in the brush-border. Functional support for the presence of a receptor was obtained recently in a study on catheterized piglets fed bovine lactoferrin (Harada *et al.*, 1999). Intact lactoferrin was found in blood and bile and histochemistry showed endocytosis by the intestinal epithelial cell. Kawakami and Lönnerdal (1991) reported the presence of lactoferrin receptors in the brush-border membranes of

both fetal and infant human small intestine. Binding was pH dependent, with optimum binding occurring at pH 6.5–7 and the apparent K_d was 1 μM. Enzymatic deglycosylation of lactoferrin did not inhibit binding, indicating that the glycan chains were not structurally involved in receptor binding and instead may contribute to the structural integrity of lactoferrin during digestion.

10.5.2 Lactoferrin Receptors in the Monocyte/Macrophage System

Lactoferrin is known to have several effects on inflammatory and immune responses of an animal during which there is a significant increase in circulating levels of lactoferrin. In most cases, the target cell is a member of the monocyte/macrophage system. This implies that lactoferrin interacts with the monocytic cells through a receptor-like mechanism. Human monocytes were shown to bind lactoferrin with high affinity (4.5×10^{-9} M) (Birgens *et al.*, 1983), virtually independent of temperature (in the range 0–37°C), but to some extent dependent on the presence of Ca^{2+}. Lactoferrin binding to other cells of the monocyte/macrophage line, namely adherent mononuclear cells (Bennett and Davis, 1981) and alveolar macrophages (Campbell, 1982), occurs at a lower affinity, the apparent K_d being 2.7×10^{-6} M for adherent cells, and 1.7×10^{-6} M for mouse peritoneal cells. The specificity of lactoferrin binding to the above cells was demonstrated in competitive binding experiments with human transferrin, monomeric and aggregated IgG, bovine albumin, and cytochrome c, as none of these proteins was shown to be competitive.

10.5.3 Characteristics of the Lactoferrin Receptor

The human intestinal receptor was isolated and partially characterized (Kawakami and Lönnerdal, 1991). Gel electrophoresis indicated a molecular weight of 115 kDa under nonreducing conditions and 38 kDa under reducing conditions. The receptor is glycosylated, the molecular weight of the glycan moiety being 4 kDa. The purified receptor maintained its ability to bind human lactoferrin as shown by ligand blotting. Mazurier *et al*. (1989) isolated a putative lactoferrin receptor from phytohaemagglutinin-stimulated human lymphocytes and reported the presence of two proteins with molecular weights of 100 and 110 kDa.

The gene for the intestinal lactoferrin receptor has been cloned and the protein has been shown to mediate internalization of lactoferrin into small intestinal Caco-2 cells (Suzuki *et al.*, 2001). This internalization of lactoferrin requires only the amino acid sequence/structure from the N-terminal 1–90 amino acids of lactoferrin, as shown by human lactoferrin-bovine transferrin chimera studies (Suzuki *et al.*, 2008). The intestinal lactoferrin receptor is also expressed abundantly during infancy (Lopez *et al.*, 2006), suggesting a crucial role for various functions of lactoferrin, but its regulation remains to be established. Recently, intestinal lactoferrin receptor gene expression was shown to be partly regulated by microRNA-584, which mediates post-transcriptional expression of intestinal lactoferrin receptor mRNA by a combination of translational repression and mRNA degradation, and also suggests an association of miRNA-584 with perinatal expression of the small intestinal lactoferrin receptor (Liao and Lönnerdal, 2009).

It is unknown whether the protein described in the cells outlined above represents a common lactoferrin receptor. Although the protein has been documented and partially characterized in terms of the physical parameters of binding, its full biological significance remains to be determined.

10.6 Implications and Significance

It is evident that there is much support for lactoferrin having several physiological roles, although firm evidence in vivo is still lacking, particularly in the human. Studies on humans have been severely limited because of a lack of adequate

quantities of lactoferrin for long-term clinical trials. Although some studies have been performed with bovine lactoferrin, it is quite possible that species-specific lactoferrin is needed for these functions. The production of recombinant human lactoferrin will make it possible to evaluate several of the above-mentioned biological activities of lactoferrin. However, it should be cautioned that the recombinant forms of lactoferrin always will have somewhat different glycan composition and this feature of lactoferrin is involved in cellular recognition or stability/turnover of the molecule; such studies may still not reveal the full range of activities exerted by lactoferrin. However, results to date suggest that at least cellular recognition of the lactoferrin molecule is not affected by the presence/absence of the glycans. It can be envisioned that the next few years will provide much-needed and interesting information on lactoferrin and its biological functions. These studies will give directions for possible applications of lactoferrin in therapy.

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Minor Proteins, Including Growth Factors

11

P.C. Wynn and P.A. Sheehy

11.1 Introduction

The processes involved with the evolution of placental mammals from oviparous species have entailed the replacement of the vitellogenin of the egg with a vascularised chorioallantoic placenta to deliver nutrients and growth regulatory molecules to the foetus. The maternal influence on the neonate is then extended through the provision of milk designed to direct the development of the young to independence in an external environment very different to that of the uterus.

Monotremes, with their abdominal milk patch together with their small egg, provide the evolutionary link with the more complex lactational strategies found in marsupial species and then the simpler versions in mammalian and primate species (Brawand *et al.*, 2008).

While milk provides the sole source of nutrients for growth, the way in which these nutrients are utilised to develop the neonate through weaning and maternal independence is also orchestrated by an abundance of small proteins mostly in the whey component. Peptides encrypted within and released from the caseins through enzymatic hydrolysis in the gastrointestinal tract are also important in directing developmental processes (Meisel, 2005). The main proteins in milk, α_{S1}-casein and β-casein, have the capacity to be processed into 20,000 separate peptides through enzymatic digestion or microbial processing (Hayes *et al.*, 2007; Hernandez-Ledesma *et al.*, 2007b). The biological roles for these peptides remain a challenge for biotechnologists to develop rapid high-throughput screening assays for novel biological activity within these peptide populations.

The importance of the impact of environmental factors on the functionality of the mammary epithelium through epigenetic modification of DNA methylation and histone modification of gene expression now opens up a whole new area whereby the bioactive protein components of milk may be modified during the key developmental windows of puberty, pregnancy and involution during which mammary tissue undergoes significant morphological and functional change (Topper and Freeman, 1980; Rijnkels *et al.*, 2010). The disruption of α_{S1}-casein synthesis induced by the lactogenic endocrine complex of prolactin, insulin and hydrocortisone through infection with *Escherichia coli* and *Streptococcus uberis* resulted from an increase in the methylation of the far upstream promoter of the bovine α_{S1}-casein-encoding gene and tighter packing of DNA chromatin (Vanselow *et al.*, 2006; Gunther *et al.*, 2009). Due to this, changes were induced in the genes encoding the chemokines, interleukins, β-defensins, serum amyloid A and

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haptoglobin so important to the inflammatory response (Gunther *et al.*, 2009), and some of which may be expressed in milk.

The refinement of techniques for assessing simultaneous gene expression of the complete genome within a tissue and then the secretory products of that tissue through the highly sensitive techniques of 2D SDS gel electrophoresis associated with mass spectrometry to identify individual proteins has allowed researchers to identify many novel proteins in milk. This technique has been used to great effect to identify minor milk proteins with an emphasis on those protecting the host from infection (Smolenski *et al.*, 2007); these studies successfully identified half of the 363 distinct spots from which 15 proteins were identified that play a role in host defence. Minor proteins associated with signals from the mammary stromal and epithelial cells that suppress the cancer phenotype in normal regenerating mammary tissue may have therapeutic application to prevent or reverse tumorigenesis (Booth *et al.*, 2011). Lactoferrin, for example, induces apoptosis while at the same time inhibiting angiogenesis, modulating carcinogen-metabolising enzymes and acting as an iron scavenger (Parodi, 2007).

Given that the neonate obtains its humoral immunity through the immunoglobulin fraction of colostrum and is dependent on the evolution of an efficient vascular system and a mature gastrointestinal epithelium to deliver nutrients to growing tissues, it stands to reason that milk will contain regulatory proteins to promote each of these processes. Currently identified proteins and their roles are reviewed here.

11.2 Vascular System

11.2.1 Angiogenins

The angiogenins comprise a small group of monomeric proteins from the pancreatic ribonuclease superfamily with a molecular weight of 14 kDa. The family consists of proteins with similar structural and catalytic elements that retain varying degrees of enzymatic activity. However, their primary sequences have diverged resulting in novel bioactivities (Dyer and Rosenberg, 2006). All of the mammalian superfamily members are extracellular proteins sharing a disulphide-bonded tertiary structure. However, the T1 RNase is found only in bacteria and fungi, while T2 RNase is ubiquitous having been identified in microbia, plants, viruses, animals, including humans. Recent evolutionary studies suggest that these proteins may have been involved initially in vertebrates as host defence or angiogenic proteins (Sorrentino, 2010).

Possibly three angiogenins have been identified in bovine milk: the original characterisation of the human angiogenin (Fett *et al.*, 1985; Strydom *et al.*, 1985) leads to the detection of two angiogenic homologues in bovine milk (Strydom *et al.*, 1997) and then two similar proteins with molecular weights of 15 kDa (presumed to be angiogenin 1 or ANG-1) and a related 17 kDa protein named lactogenin or ANG-2 (Ye *et al.*, 1999). Bovine ANG-2 differs from ANG-1 by the presence of a single glycosylation site which results in a reduction in its ribonuclease activity compared with ANG-1. The differences between ANG-1 and ANG-2 (there is a 57% sequence homology between them) incorporate a recognition sequence for an integrin receptor which may be implicated in the development of the vasculature (Strydom *et al.*, 1997).

The first discovered of these vasculature development molecules was human angiogenin, which has been shown to be effective across species, including the chicken chorioallantoic membrane, rabbit cornea and rabbit knee meniscus. The link between the ribonuclease activity and angiogenic capacity of these proteins seemed tenuous until site-directed mutagenesis studies of the catalytic domain of murine angiogenin 4 showed that this enzymatic activity was essential for this process (Crabtree *et al.*, 2007).

Angiogenin was also shown to trigger nitric oxide synthase (NOS) activity in human umbilical vein endothelial cells and embryonic stem cell-derived endothelial cells independently from its RNase activity (Trouillon *et al.*, 2011).

Angiogenin modification of engrafted mesenchymal stem cells enhanced their tolerance to hypoxia injury *in vitro* and improved their

viability in infarcted hearts, thus helping preserve the left ventricular contractile function and attenuate left ventricular remodelling through vasculogenesis (Liu *et al.*, 2008). Thus, these proteins are capable of altering vascular function in a variety of ways.

The angiogenins appear to play a role beyond the vascular system in the nervous system: key mutations are associated with familial as well as sporadic forms of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder causing selective destruction of motor neurons (McLaughlin *et al.*, 2010).

Bovine angiogenin is also mainly responsible for the inhibitory effect of bovine milk on osteoclast-mediated bone resorption through a direct effect on osteoclasts (Morita *et al.*, 2008).

Bovine milk angiogenin also induces the production of cytokines IL-1β, IL-6 and TNF-α in human leukocytes (Shcheglovitova *et al.*, 2003) and therefore plays a role in host defence.

It is important to recognise that angiogenesis is a complex process whereby new blood vessels form from pre-existing vasculature in response to proangiogenic factors such as basic fibroblast growth factor (bFGF) and the 165 kDa isoform of vascular endothelial growth factor (VEGF165): antiangiogenic factors have also been identified. The pepsin-derived N-terminal fragment of the iron- and heparin-binding protein lactoferrin (LfcinB), initially thought to exert antimicrobial properties only (Bellamy *et al.*, 1992), also blocks development of the vasculature. It interferes with bFGF- and VEGF165-induced angiogenesis by competing successfully for binding sites on vascular endothelial cells (Mader *et al.*, 2006). This explains the antimetastatic role identified for this protein (Yoo *et al.*, 1997).

11.2.2 Angiotensin-Converting Enzyme Inhibitory Peptides

Angiotensin-converting enzyme (ACE) is responsible for the conversion of angiotensin 1 to active angiotensin 2 and the degradation of the vasodilator, bradykinin. While many of the angiogenic milk proteins are multifunctional, the role of milk-derived peptides in inhibiting ACE is well established (Ricci *et al.*, 2010). The commercial significance of these peptides to the health-giving properties of milk cannot be underestimated given that a decrease in diastolic pressure of just 5 mmHg in hypertensive patients could potentially reduce the incidence of cardiovascular disease by 16% (Unger, 2002). While the renin-angiotensin system is critical for blood pressure control, the kinin-nitrous oxide pathway, the neuroendopeptidase system and the endothelin enzyme regulatory system also contribute to its integrity (Weber, 2001).

The extensive literature on ACE inhibitory peptides in milk has been reviewed perceptively by Ricci *et al.* (2010). The major contributors to the extensive list of bovine ACE inhibitory peptides are α_{S1}-casein with 19 peptides varying in length from 3 to 30 amino acids and β-casein with 18 peptides, while enzyme hydrolysis resulting in milk serum generated 18 peptides from β-lactoglobulin and 4 from α-lactalbumin. The enzymatic digestion of milk has yielded an additional 37 peptides from β-casein, 2 from α_{s1}-casein and 2 from β-lactoglobulin with hypotensive activity (Ricci *et al.*, 2010).

Some of these peptides derived from β-lactoglobulin also exhibit radical-scavenging activities. These antioxidative effects also increase the health-giving potential of milk (Hernandez-Ledesma *et al.*, 2007a).

De novo synthesis of ACE inhibitory peptides in the mammary epithelium would appear to be unwarranted given the diversity of sources of hypotensive peptides available from milk consumed through both the casein and whey milk components. However, this very diversity demonstrates how important the control of blood pressure has become in the evolution of contemporary species. It also highlights the fact that dietary peptides will be altered dramatically through enzymatic hydrolysis *en route* to the circulation and therefore the target tissue depending on pH in the stomach or abomasum in ruminants. Their level of absorption from the small intestine and then their resistance to further processing by circulating peptidases is important. Thus, the beneficial effects of functional foods supplemented with

ACE inhibitory peptides (Murray and Fitzgerald, 2007) should be evaluated carefully since the delivery of the peptides to their site of action is essential.

From a physiological sense, it is interesting that the vasoregulatory potency of these peptides varies by over 1,000-fold with the most potent being a hexapeptide derived from goat α_{s2}-casein having an IC50 value of around 2.4 μM (Quiros *et al.*, 2005). By contrast, the functional significance of the lowest affinity peptides must be evaluated carefully.

The necessity for a diversity of functional ACE inhibitory sequences may also result from the differences in the functionality of the two catalytic domains of ACE. *In vivo*, most ACE activity on angiotensin 1 is induced by the ACE C-domain. However, the antifibrotic peptide AcSDKP, angiotensin 1-7 and amyloid β protein 1-42 are substrates for the N-domain only (Bernstein *et al.*, 2011). AcSDKP is an intriguing peptide in that it was described initially as a regulator of haematopoietic stem cell proliferation (Bonnet *et al.*, 1992), but it also prevents the proliferation of fibroblasts in the myocardium, aorta and kidney subjected to insult or injury (Peng *et al.*, 2003; Lin *et al.*, 2008; Liao *et al.*, 2010). Clearly, the promotion of these bioactivities through the consumption of milk proteins provides a compelling marketing tool for milk.

11.2.3 Heparin Affin Regulatory Peptide

Heparin affin regulatory peptide (HARP) is a 136-amino acid (18 kDa) growth factor with a high affinity for the anticoagulant glycosaminoglycan heparin. It has been assigned a number of names, including pleiotrophin, heparin-binding growth-associated molecule, heparin-binding growth factor 8 and heparin-binding neurite-promoting factor, and was identified initially in the brain with a role in regulating neurite growth (Rauvala, 1989). It is also expressed in the heart, uterus, cartilage, bone and the mammary gland (Bernard-Pierrot *et al.*, 2004). It is secreted into both human colostrum and milk, with threefold higher concentrations being found in colostrum. It is found in association with heparin sulphate and dermatan/chondroitin sulphate glycosaminoglycans in the extracellular matrix of many tissues and influences epithelium-mesenchyme interactions and neuronal migration during development (Bernard-Pierrot *et al.*, 2004).

As with other proteins expressed in milk, HARP has been implicated in a number of physiological functions. It is capable of transforming the phenotype of cell lines and also stimulates cell replication and chemotaxis and importantly has a key role in promoting angiogenesis as demonstrated *in vivo* and *in vitro*.

Importantly, HARP promotes angiogenesis: the intact peptide and the HARP residues 1–21 and residues 121–139 are implicated in stimulating endothelial cell tube formation on Matrigel and collagen and fibrin gels, by activating endothelial cell migration. These peptides also induce angiogenesis in the *in vivo* chicken embryo chorioallantoic membrane assay (Papadimitriou *et al.*, 2001).

The complexity of the actions of this protein has increased with the realisation that enzymatic processing in the extracellular environment yields a series of peptides with either similar or opposite actions to the parent protein (Papadimitriou *et al.*, 2010). The C-terminal fragment 122–131 inhibits cell adhesion, anchorage-independent proliferation and migration of cell lines that express HARP themselves. Importantly, it inhibits angiogenesis *in vivo* at concentrations of 2 nM and the phosphorylation of key signal transduction intermediary proteins and interferes with the activity of the HARP receptor family (Papadimitriou *et al.*, 2010). The cleavage of this peptide in milk is yet to be demonstrated. However, the potential for this peptide to provide an antitumorigenic effect in milk provides a compelling reason to investigate it.

Its role in regulating angiogenesis may also be associated with its interaction with the VEGF. HARP forms a complex with VEGF and inhibits VEGF binding to its high-affinity receptor VEGFR2, thus halting angiogenesis (Herault *et al.*, 2004). It is also used as a tumour marker and importantly is elevated in response to proinflammatory cytokines.

11.2.4 Kininogen

The kininogens are multifunctional and multidomain glycoproteins related to the cystatins (Lalmanach *et al.*, 2010). Two forms of kininogen have been isolated from bovine milk which vary in size: they comprise both high and low molecular weight forms of 68 kDa and 16–17 kDa, respectively, which differ from those identified in bovine plasma (Wilson *et al.*, 1989). The 78 kDa circulating moiety is cleaved by plasma protease, kallikrein, to release four fragments: heavy chain, bradykinin, fragment 1.2 and light chain. These have been identified in bovine milk, and the fragment 1.2 exhibits the ability to stimulate osteoblast proliferation (Yamamura *et al.*, 2000). This latter fragment is multifunctional and stimulates bone formation while at the same time inhibiting bone resorption: its biological activity is pepsin resistant (Yamamura *et al.*, 2006).

11.3 Immune Function

The newborn has a need for a wide spectrum of peptides and proteins contributing to host defence mechanisms since their innate immune function is poorly developed at birth. These peptides often act by limiting bacterial access to the intestinal mucosa. The expression of this family of milk proteins is likely to be adjusted by environmental influences during mammary gland development from foetal life to pregnancy and lactation by epigenetic means given their role in supporting neonatal health (Rijnkels *et al.*, 2010).

Lactoferrin has long been recognised as an important effector of iron transport but now is recognised as just one of the growing family of proteins that appear to have varying functions in different environments (Legrand and Mazurier, 2010; Amini and Nair, 2011; Manzoni *et al.*, 2011). Similarly, α-lactalbumin is both a calcium-binding milk protein and bactericide for gram-positive organisms after trypsin and chymosin hydrolysis in addition to its recognised role in regulating lactose synthetase activity (Yalçin, 2006; Rusu *et al.*, 2010). Both of these proteins are not generally considered minor proteins of milk and therefore are discussed elsewhere in this volume.

11.3.1 β_2-Microglobulin

β_2-Microglobulin is found in bovine milk and in other body fluids as a multimer with a molecular mass of 11.8 kDa (Hoshi *et al.*, 1996; Boehmer *et al.*, 2008). β_2-Microglobulin is a product of the digestion of the cellular component of milk and is associated with the continuous active transport of IgG into the milk of mice and the peak of IgG expression observed at parturition in the cow (Adamski *et al.*, 2000). Its role in this process may involve the regulation of the mammary Fc receptor.

β_2-Microglobulin has also been implicated in the functional integrity of cells since administration of antibodies to the protein in β_2M/MHC class I-expressing malignancies induces tumour cell apoptosis (Yang and Yi, 2010). It is also an important immunological protein across the complex lactational cycle of the tammar wallaby (Joss *et al.*, 2009). Clearly there is more to be learned about the functional requirements for this milk protein in the neonate.

11.3.2 Osteopontin

Osteopontin is an acidic protein of 262 amino acid residues, which is heavily phosphorylated and glycosylated and has an arginine-glycine-aspartic acid-binding domain as well as two heparin-binding sites, one thrombin cleavage site and a putative calcium-binding site (Standal *et al.*, 2004). It was found initially in bovine species in the mineralised matrix of bone (Franzen and Heinegard, 1985) and more recently in many tissues and fluids, including urine and milk (Senger *et al.*, 1989).

Osteopontin is involved in a number of physiological and pathological events, including angiogenesis, apoptosis, inflammation, wound healing and tumour metastasis (Lonnerdal, 2011). It is a protein that modulates immune function and stimulates Th1/Th2 switching; it also possibly

affects bone mineralisation and growth. Biological activities of lactoferrin may be facilitated by osteopontin (Lonnerdal, 2011).

Within the 262-amino acid sequence are a large number of serine or threonine residues that may be phosphorylated or glycosylated. The calculated molecular weight of the protein is 29,283 Da and includes a cell adhesion sequence (RGD) that may bind to integrins and facilitate their action.

The inflammatory response provides a key stimulus for this protein as for lactoferrin; tenfold higher concentrations of this protein relative to that for lactoferrin are found in human milk (Masson and Heremans, 1971). This protein may act as a binding protein for the transport of lactoferrin thus altering its kinetics of clearance and therefore its biological effectiveness as a potent antibacterial, antifungal, antiviral, antitumour and anti-inflammatory agent (le-Grande *et al.*, 2008). This contention is supported by calorimetry studies of the interaction between these two proteins in which the regions of electrostatic complementarity between OPN and LF were identified which mediate the numerous biological functions of each protein (Yamniuk *et al.*, 2009). These two genes were also co-induced at involution in the mouse (Baik *et al.*, 1998).

Complexity interactions between minor milk proteins can influence their function in milk; the anti-inflammatory actions of the milk fat globule-epidermal growth factor-8 protein (MFG-E8), for example, are modulated through the binding of cell-surface integrins to osteopontin which becomes activated during the gastrointestinal inflammatory disease, colitis (Aziz *et al.*, 2009).

Osteopontin also plays a role in mammary involution as well as casein synthesis. The osteopontin transcript SPP1 is increased during involution in mammary tissue while its suppression using siRNA technology decreases casein gene expression in bovine primary mammary epithelial cells (Sheehy *et al.*, 2009). Its immunological role is amply demonstrated by the association between specific polymorphisms in the SPP1 gene and the somatic cell score in mastitis in bovine mammary tissue (Alain *et al.*, 2009).

11.3.3 Proteose Peptone 3

Proteose peptone 3 is one of a family of phosphorylated glycoproteins in the proteose peptone component of the whey fraction of milk, is not derived from the proteolysis of casein and has an apparent molecular mass of 28 kDa; it is composed of 135 amino acid residues (Sorensen and Petersen, 1993). The Ser residues at positions 29, 34, 38, 40 and 46 in the amino acid sequence are all phosphorylated. One N-linked carbohydrate group is found at Asn_{77}, while *O*-linked carbohydrate groups are located at Thr_{16} and Thr_{86}: furthermore Thr_{16} is only approximately 50% glycosylated. The amino sugar found at Thr_{86} provides a linkage for either galactosamine or glucosamine. In contrast, both glucosamine and galactosamine are found in the carbohydrate group linked to Asn_{77} (Girardet and Linden, 1996).

More recent analyses using a panel of hydrophobic absorbents have shown that high and low molecular weight forms of this peptide exist in milk (Sousa *et al.*, 2008). This peptide belongs to the glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) family (Girardet *et al.*, 2000) and could therefore play an immunological role in the suckling young particularly in relation to possible interactions with enteric bacteria. Other related proteins include glycomacropeptide, lactoferrin and κ-casein.

11.3.4 Lactoperoxidase

Lactoperoxidase plays a major role in regulating enteric bacteria in the neonate through its expression in colostrum. The enzyme catalyses the oxidation of thiocyanate (SCN^-) in the presence of H_2O_2 to produce an intermediate product with antimicrobial properties (Visalsok *et al.*, 2004). Lactoperoxidase is expressed at surprisingly high levels with 11–45 mg/L in colostrum and 13–30 mg/L in milk (Korhonen, 1977).

The indigenous lactoperoxidase in milk may be exploited for the cold sterilisation of milk, while the isolated enzyme may be added as a bactericidal agent to milk replacers for young calves

or piglets. Lactoperoxidase may be a useful additive for infant formulae, perhaps because human milk contains very little or none of this enzyme (Chap. 12; Fox, 2001).

11.3.5 Lysozyme

Lysozyme is an important enzyme in the animal's host defence system. It catalyses the hydrolysis of the β1–4 linkages between *N*-acetyl muramic acid and *N*-acetyl glucosamine in the peptidoglycan layers of the bacterial cell wall (Johnson, 1994; Taylor and Leach, 1995). It is active against most Gram-positive bacteria, particularly the thermophilic sporeformers. It is found in colostrum and normal milk at 0.14–0.7 mg/L and 0.07–0.6 mg/L, respectively (Korhonen, 1977).

As with many of these host defence factors, it acts in conjunction with lactoferrin to neutralise *E. coli* with lactoferrin initially altering the outer membrane of the Gram-negative bacteria to render it susceptible to enzymatic proteolysis by lysozyme (Severin and Wenshui, 2005). The potential of this enzyme has now been realised with the development of transgenic pigs and cattle expressing concentrations that are 50-fold higher than normal endogenous milk levels: in the case of the cattle, expressed levels were as high as 25 μg/mL (Chap. 12; Tong *et al.*, 2011; Yang *et al.*, 2011).

11.3.6 TGFβ1 and 2

A number of the cytokines have been identified in milk from different species.

Transforming growth factor (TGF)β1, interleukin (IL)-4 and IL-10 are all found in human milk and are expressed at higher levels in an allergic response (Marek *et al.*, 2009). TGFβ1 and 2 are expressed at levels of 400 ng/mL and over 3 μg/mL in bovine milk, respectively (Savilahti *et al.*, 2005). Milk protein synthesis is suppressed by exogenous TGFβ1 during gestational development of the gland but not during lactation. Consistent with reports linking TGFβ1 gene expression with mammary gland involution following lactation, over-expression of TGFβ1 in the differentiating secretory epithelium leads to premature programmed cell death in the absence of a negative effect on secretory epithelial cell proliferation (Smith, 1996). The expression of these cytokines is increased in response to *E. coli* mastitis (Chockalingam *et al.*, 2005) and decreased by half after the heating process of pasteurisation (Peroni *et al.*, 2009). Clearly these cytokines form an integral part of the host defence system.

11.4 Gastrointestinal Tract

One of the key developmental functions served by colostrum initially and then milk is to facilitate the maturation of the gastrointestinal epithelium to ensure the efficient absorption of the nutrient supply to support growth processes (Blum and Baumrucker, 2008). This rich source of growth factors is mitogenic in many cell lines associated with the gastrointestinal tract (Belford *et al.*, 1995). Bioactive peptides, however, need to be resistant to the acidic conditions (pH 3–5) and pepsin activity in the stomach of the neonate to exert their effects. In some instances the bioactivity of a peptide can actually increase in this environment: for example, digestion of TGFβ with pepsin at pH 2 or 3.5 and with pancreatin will increase the concentrations of this peptide in human milk (Lonnerdal, 2010).

11.4.1 IGF-1 and IGF-2

The insulin-like growth factor (IGF) complex of peptides and binding proteins is well represented in colostrum and milk. The IGF-binding proteins (IGFBPs) are a family of six homologous proteins with high binding affinity for IGF-1 and IGF-2. There are also five binding proteins with a tenfold lower affinity; IGF binding may be modulated by IGFBP modifications, such as phosphorylation and proteolysis, and by cell or matrix association of the IGFBPs. All six IGFBPs have been shown to inhibit IGF action, but stimulatory effects have also been established for IGFBP-1,

IGFBP-3 and IGFBP-5 which are independent of type 1 IGF-1 receptor signalling. IGFBP-1 exerts these effects by signalling through $\alpha_5\beta_1$-integrin, whereas IGFBP-3 and IGFBP-5 may have specific cell-surface receptors with serine kinase activity (Baxter, 2000).

These binding proteins can interact with the signalling pathways for other growth factors: for example, IGFBP-3 interacts with TGFβ signalling through Smad proteins and also influences other signalling pathways (Firth and Baxter, 2002). Lactoferrin likewise competes with IGF-1 for binding to IGFBP-3 and modulates the role of the IGF system in involution (Baumrucker and Erondu, 2000).

Initially the IGF system was thought to mediate the galactopoietic influence of somatotropin thereby supporting the classic somatomedin hypothesis. IGF-1 is synthesised in mammary stromal cells but not epithelial cells: by contrast, IGF-2 is synthesised in the bovine mammary epithelium (Baumrucker *et al.*, 1993).

The clear demonstration that IGFBP-3 expression increases in the circulation and in milk with advancing lactation suggests that the IGF complex plays an important role in the process of involution (Gibson *et al.*, 1999), although milk IGFBP-3 proteases (Lamson *et al.*, 1991) modulate this role. There is still much to learn about the role of this growth factor complex in milk.

11.4.2 EGF and TGFα

Another peptide growth factor with a central role in directing the differentiation and proliferation of epithelial cells in the gastrointestinal tract is epidermal growth factor (EGF). Initially isolated from the submaxillary salivary glands of mice and recognised for its unique ability to stimulate tooth eruption and eyelid opening (Cohen, 1962), this peptide is expressed at high levels (up to 200 ng/mL) initially in human colostrum which then drops substantially in 7 days: in contrast its homologue TGFα has been found at a 100-fold lower concentration but does not vary markedly during early lactation (Okada *et al.*, 1991). Concentrations in bovine milk appear to be much lower, around 2–3 ng/mL (Iacopetta *et al.*, 1992). A further member of the EGF family, betacellulin, is also found in bovine milk at similar low but still physiological concentrations (Bastian *et al.*, 2001). However, bovine milk may also contain specific inhibitors of EGF degradation (Rao *et al.*, 1998), suggesting that these lower levels are physiologically relevant.

11.5 Binding Proteins

11.5.1 Folate-Binding Protein

Folate-binding proteins (FBPs) are ubiquitous, soluble and membrane-bound high-affinity receptors for folate, an essential nutrient involved in nucleic and amino acid metabolism (Heegaard *et al.*, 2006). The proteins occur in isoforms equipped with a hydrophobic glycosylphosphatidyl inositol tail, enabling anchorage to plasma membranes as a membrane-bound folate receptor (Holm and Hansen, 2003). Folate appears to be important in the regulation of milk protein synthesis across species including the cow, Cape fur seal and Tammar wallaby (Menzies *et al.*, 2009). FBP is typically found in milk as a monomer, exhibiting a molecular weight of approximately 25,720 Da, but with its carbohydrate component, this increases to 30 hDa (Svendsen *et al.*, 1984). The 222-amino acid protein has up to eight disulphide bonds and is glycosylated at two amino acids. It has a role in sequestration of folate to facilitate cellular uptake including in the gastrointestinal tract. Folate is important for embryonic development since a deficiency may be implicated in neural tube defects like spina bifida (Copp and Greene, 2010). FBP may also fine-tune the availability of this vitamin from milk for different tissue functions and sequester it from gastrointestinal microflora (Urquhart *et al.*, 2010).

11.5.2 Vitamin D-Binding Protein

Calcium in human milk is regulated indirectly by regulating the concentration of citrate and casein

in the milk (Neville *et al.*, 1994). Calcium flux during lactation is important in the development of the casein micelle. While vitamin D promotes intestinal calcium absorption, it has no known effect on calcium transport across the membrane of the Golgi apparatus (Kent *et al.*, 2009). The vitamin D sterol family is transported by a specific vitamin D-binding protein (DBP), which is a polymorphic serum glycoprotein of 52 hDa (458 amino acids) that also binds G-actin, fatty acids and certain chemotactic agents. It has been detected in the whey of colostrum and blood serum at much higher concentrations than is found in mature milk (Swamy *et al.*, 2002). These proteins are of comparable size from human, monkey, porcine and bovine sources although the bovine seems to lack the smaller binding protein (Hollis and Draper, 1979). Other milk proteins have also been indicated in vitamin D transport in milk.

11.5.3 Vitamin B12-Binding Protein

Vitamin B12 (cobalamin) is important for ruminant species to potentiate the conversion of glucose to succinate in the liver. The concentration of endogenous cobalamin in cows' milk is 3.3 nM, while the cobalamin-binding capacity of serum is 0.05 nM. Cobalamin is distributed between a 280 kDa protein complex (45%) and a 43 kDa cobalamin binder (55%) in cow's milk (Fedosov *et al.*, 1996).

This vitamin, along with a number of analogues, which are structural isoforms, is synthesised by bacteria. Vitamin B12 is not readily available across the placenta or from milk of ruminants, nor are injected sources of these vitamins retained. This vitamin is obtained from dietary sources in nonruminant species. The human vitamin B12-binding protein (haptocorrin) has a molecular weight of approximately 43 kDa (Brada *et al.*, 2000) and is heavily glycosylated (34% carbohydrate). This vitamin transporter assists with the absorption of vitamin B12 in young infants (Lonnerdal, 2010) and limits its access for microbial uptake and thus may influence the growth of gastrointestinal microflora (Gullberg, 1973).

11.5.4 Riboflavin-Binding Protein

Riboflavin-carrier (or binding) protein is an oestrogen-inducible phospho-glycoprotein (M_r 37 kDa) required in egg-laying vertebrates for yolk deposition of the vitamin to support growth and development of the prospective embryo (Adiga, 1994). The vitamin carrier is evolutionarily conserved in mammals including subhuman primates and humans (Adiaga *et al.*, 1997) and plays a pivotal role in embryonic development during gestation. The protein in milk, which is approximately 37 kDa in mass, is most likely sequestered from plasma and binds riboflavin (Jenness, 1974). The concentration in mammalian milk is higher than in the circulation during lactation. It is therefore attractive to hypothesise that the riboflavin-binding protein is synthesised by the lactating mammary gland in response to oestrogen to sequester the vitamin for secretion into milk for neonatal nutrition (Karande *et al.*, 2001). This carrier modulates the function of this vitamin in fetal development. Interestingly this protein bears a remarkable similarity (30% of sequence) to the chicken FBP, with eight of the nine disulphide bonds conserved between the two proteins; clearly there is an important conservation of sequences for these transport proteins (Zheng *et al.*, 1988).

11.6 Mammary Gland and Maternal Physiological Regulatory Function

The biological influence of milk-borne minor proteins and growth factors is not restricted to impact on the growth, development, metabolism and immuno-protection of the neonate as there are also autocrine and paracrine influences of these minor milk constituents on the maternal physiology, most notably directly impacting the function of the mammary gland itself. Some of these interactions are described below.

11.6.1 Leptin

Leptin is a peptide hormone initially noted as being secreted from adipose tissue and playing a

role in energy metabolism. Leptin has also been identified as being expressed in bovine mammary epithelial cells and the level of expression responsive to stimulation by insulin, IGF-1 and prolactin (Smith and Sheffield, 2002; Feuermann *et al.*, 2004). This has led to speculation that milk leptin may be derived from mammary epithelium rather than maternal circulation and may have some role in neonatal metabolism. Importantly, leptin receptors have also been identified in the mammary epithelium (Feuermann *et al.*, 2004), suggesting a potential autocrine or paracrine role in mammary tissue. A more recent report, however, has suggested that leptin modulation of mammary epithelial cells is mediated by leptin derived from the adipocytes or mammary fat pad in cows (Feuermann *et al.*, 2006). The regulation by insulin and IGF-1 has also led to consideration of roles for leptin in nutrient partitioning: importantly the prolactin response occurs only in tissue culture of lactating mammary tissue and not in tissue isolated from calves. In another report utilising bovine mammary epithelial cells from a pregnant heifer, GH and the lactational hormone complex of insulin, prolactin and dexamethasone resulted in suppression of leptin mRNA expression, further complicating the role this peptide may have in lactational regulation (Yonekura *et al.*, 2006).

Interestingly, leptin added to bovine mammary epithelial cell culture inhibited proliferation in a dose-dependent manner and was suggested to play a role in impairment of mammary development in a proportion of prepubertal heifers fed high-energy diets, although this is most likely under the influence of serum-derived leptin (Silva *et al.*, 2002). In contrast, a report by Feuermann *et al.* (2008) identified that the coculture of leptin and prolactin with mammary epithelial cells enhanced proliferation of cells, possibly through reduced apoptosis: thus leptin may potentiate the actions of prolactin on bovine mammary epithelial cells in culture.

Leptin is expressed more highly in bovine colostrum than in mature milk, but at a higher concentration in milk than in plasma at comparable stages of lactation (Pinotti and Rosi, 2006; Parola *et al.*, 2007). While not necessarily directly relevant to the expression of leptin in milk, allelic variation in a specific single nucleotide polymorphism (Arg25Cys C to T) in the leptin gene where animals are homozygous for the T allele results in them producing higher milk yields across lactation (Buchanan *et al.*, 2003).

11.6.2 FIL

The feedback inhibitor of lactation (FIL) is a whey protein of ~7.6 kDa (caprine) secreted into milk of many species and accumulates in the mammary gland in the interval between milk removal (Knight *et al.*, 1998). It seemingly has an autocrine/paracrine effect on mammary epithelial cells which in turn regulates milk biosynthesis and secretion. The co-incubation with purified goat FIL and murine mammary epithelial cells *in vitro* indicated that while some inhibition of milk protein synthesis could be observed, FIL also inhibited the secretion of existing intracellular milk protein as well as exerting an autocrine regulation of mammary milk biosynthesis (Rennison *et al.*, 1993). Immunisation against FIL during late lactation in goats reduced the rate of decline of lactation (Wilde *et al.*, 1996): similarly, FIL may also have a role in apoptosis in involution of the mammary gland following cessation of milk removal (Wilde *et al.*, 1999). The precise mechanisms by which FIL interacts and influences mammary epithelial cells' function are not completely defined, and there has been little research on this autocrine regulator in recent years.

11.6.3 Parathyroid Hormone-Related Protein

Parathyroid hormone-related protein (PTHrP) existing as multiple bioactive peptides has been observed in the milk of a number of species including the cow. Its expression increases over lactation (Goff *et al.*, 1991) and is loosely correlated with the calcium concentration in milk, suggesting a role for this protein in calcium transport to milk from the maternal circulation (Law *et al.*, 1991).

This was more clearly demonstrated in goats where exogenous administration of PTHrP resulted in increases in calcium, phosphorus and magnesium in milk (Barlet *et al.*, 1992). The protein is also expressed in the mammary gland itself during late pregnancy and during lactation, specifically in mammary epithelial cells (Okada *et al.*, 1996; Wojcik *et al.*, 1998). Its secretion back into maternal circulation from the mammary epithelium (determined via measurement in mammary venous sampling) may influence calcium homeostasis in the dam in goats (Ratcliffe *et al.*, 1992) and influence maternal phosphorus metabolism (Barlet *et al.*, 1993). Importantly, PTHrP has been detected in the plasma of calves after suckling but not detected at birth (Goff *et al.*, 1991) although the biological activity seems to be altered in the neonatal circulation.

As well as a role in mineral translocation to milk, PTHrP seemingly has a direct influence on mammary gland physiology, with exogenous administration of synthetic PTHrP or fragments increasing mammary blood flow in goats (Prosser *et al.*, 1994) and sheep (Davicco *et al.*, 1993). Similarly, in rat mammary epithelial cells, co-incubation with PTHrP bioactive fragments stimulates cAMP messaging suggesting a coordinated regulatory role in mammary tissue (Ferrari *et al.*, 1993). Over-expression of PTHrP in transgenic mice results in hyperplasia in mammary tissue and impairment of branching morphogenesis (Wysolmerski *et al.*, 1995).

11.6.4 Relaxin

Bovine relaxin is a 6-kDa peptide that has been observed in bovine milk and is known to have insulin-like properties. The effects of relaxin in the milk of humans, rats and pigs have been studied and reviewed by Bani (1997), but very little is known of the role of bovine relaxin in milk. In pigs and other species, relaxin is highest in early lactation (Frankshun *et al.*, 2009), although the concentration of bovine relaxin across lactation is less well defined. It is also unclear if milk relaxin is derived from maternal circulation or synthesised by mammary epithelial cells, although it has been identified as being expressed in mammary parenchyma: hystero-ovariectomy at parturition does not eliminate the presence of relaxin in milk from dogs (Goldsmith *et al.*, 1994).

Intravenous injection of relaxin did not have an appreciable effect on milk ejection in cows (Donker, 1958), although there was some influence on milk let-down in sheep (Shaffhausen *et al.*, 1954). The effect of relaxin on mammary development has been studied, and sequestration of relaxin during pregnancy by antibody administration results in malformation of nipples in rats suggesting a role for this protein in mammary development (Kuenzi and Sherwood, 1992). There has also been some indication that relaxin can influence human breast cancer cells in culture (Sacchi *et al.*, 1994a, b; Binder *et al.*, 2002).

11.7 Other Minor Milk Proteins and Growth Factor of Various Function

There are additional minor milk proteins which are consistently present in bovine milk or the milk of other species, yet their specific physiological roles are less clearly defined. The roles of some of these minor protein constituents of bovine milk follow.

11.7.1 Mucins and Other Glycoproteins

Bovine milk mucins are a series of highly glycosylated glycoproteins that have been identified in milk, with bovine mucin 1 (MUC1 also known as PAS1) being a major constituent of the milk fat globule membrane (MFGM). Similar mucins have also been identified in human and mouse tissues and milk. Bovine MUC1 contains 50–60% carbohydrate by mass and has been investigated by a number of researchers for its ability to inhibit pathogen infection of epithelial surfaces. Bovine MUC1 has been shown to inhibit infection by some retroviruses (Kvistgaard *et al.*, 2004) and has been shown to bind to *E. coli in vitro* (Sando *et al.*, 2009). Murine MUC1-deficient mice were

able to be colonised by fivefold more *Helicobacter pylori* on day 1 of infection compared to normal mice of the same genotype (McGuckin *et al.*, 2007).

Multiple bovine MUC1 transcripts have been identified, differing in the number of variable tandem repeat regions expressed (Rasero *et al.*, 2002; Sando *et al.*, 2009): these transcripts have also been identified in primary and immortalised bovine mammary epithelial cells in culture (Strandberg *et al.*, 2005). Different polymorphic forms of bovine MUC1 have also been assessed as a marker for productive characteristics in dairy cows, but little association was found (Hens *et al.*, 1995). The lectin-binding properties and therefore the carbohydrate characteristics of milk-derived bovine MUC1 have also been determined (Liu *et al.*, 2005).

Bovine mucin 15 (MUC15) has also been identified and characterised in bovine milk. It has a mature peptide of 307 amino acid residues and has both O- and N-glycosylations (Pallesen *et al.*, 2002) and has previously also been identified as PASIII glycoprotein C, glycoprotein 4, component II and PAS3. Clearly a minor protein, it constitutes approximately 0.08% of total protein of milk by weight and only 1.5% by weight of the protein associated with the MFGM (Pallesen *et al.*, 2007). The extensive carbohydrate component of the total weight has been characterised (Pallesen *et al.*, 2007).

As well as those mucins described above, there are a number of glycoproteins that have been identified in the bovine MFGM. These have been identified using a variety of different names and shown to have a number of potential bioactivities, some of which have been reviewed by Mather (2000) and their potential application as nutraceuticals by Spitsberg (2005).

PAS6 and 7 are some of the more abundant MFGM glycoproteins, and their chemical characteristics have been described (Hvarregaard *et al.*, 1996). Importantly, this report also identifies the presence of two EGF-like domains in both proteins as well as some sequence homology with blood clotting factors inferring specific biological functions. Yet the role of these in milk is unclear and may well relate more to that of glycoproteins secreted from other glandular tissues.

Bovine CD36 (PASIV), with the MFGM form, has a molecular weight of ~76–78 kDa, and its detailed glycobiology has been characterised (Greenwalt and Mather, 1985). Further, Greenwalt *et al.* (1992) discuss the role of this glycoprotein in cell adhesion and signal transduction in a variety of cell types, yet its role in milk is unclear.

11.7.2 Bovine Serum Albumin

While the role of bovine serum albumin (BSA) is well understood within the circulation of the cow, its role in milk is less well defined. While many consider that the presence of BSA is due to the disruption in maternal mammary epithelia cell ultrastructure, this 66,433 Da (583 amino acid residue) protein is typically found in bovine milk. An acute increase in BSA has been observed in response to mastitis (Harmon *et al.*, 1976) and has been evaluated by several investigators as a potential marker for mastitis although the natural between animal variations is such that it is a poor indicator. BSA concentration in milk seemed to alter with stage of lactation (Sheldrake *et al.*, 1983) with the relative concentration of BSA per millilitre of milk increasing as milk yield declined in late lactation (Guidry *et al.*, 1980). This relationship between yield and relative concentration of BSA in milk is also evident with cows milked once daily compared to twice daily: as milk yield declines, relative BSA concentration increases. Feed restriction, however, suppressed mean BSA yield in milk (Lacy-Hulbert *et al.*, 1999). BSA has been identified as a significant allergen in children (Restani *et al.*, 2004). Similarly, there is also a hypothesis that the BSA present in milk may stimulate an immune response in humans that is cross-reactive with a pancreatic β-cell-specific surface antigen; this may evoke an autoimmune response potentially leading to insulin-dependent diabetes although the specific causal relationship is yet to be definitively demonstrated (Persaud and Barranco-Mendoza, 2004).

11.8 Concluding Remarks

The array of minor milk proteins and growth factors that are found in bovine milk elicit significant effects not only on the growth and development of the neonatal calf but also on the maternal physiological regulation of lactation, neonatal and dam immune function and protection from infection. Clearly, those molecules synthesised and secreted from the mammary gland are of significance, but importantly a number of molecules secreted into milk either actively or passively through tight junctions between mammary epithelial cells from the maternal circulation also play an important regulatory role; clearly these molecules are not simply chance milk constituents but are present in bovine milk to fulfil specific regulatory roles. A clear example of this evolution of secretion from the maternal circulation is the role of maternal immunoglobulins in colostrum (discussed in Chap. 9) which has long been accepted as a deliberate biological mechanism. Evidence presented here suggests that other molecules like BSA and other growth factors are also present in milk as part of the developmental regulatory spectrum.

This review is not intended to be exhaustive but rather to provide some insight into the major roles of minor milk proteins and growth factors; as the technology to test for these molecules in milk evolves, it is clear that more minor proteins and peptides that evoke biological responses in the neonate as well as the maternal physiology will be identified and characterised.

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Indigenous Enzymes of Milk

12

J.A. O'Mahony, P.F. Fox, and A.L. Kelly

12.1 Introduction

The indigenous enzymes in milk have been the subject of considerable research for 130 years. To date, about 70 indigenous enzymes have been reported in normal bovine milk (see Fox et al., 2003). With the exception of lipoprotein lipase (LPL) and xanthine oxidoreductase (XOR), most of the indigenous enzymes in milk have no obvious physiological role in the biosynthesis and secretion of milk, and only a few have an obvious function in milk post-secretion. LPL hydrolyses triglycerides in the chylomicrons in blood and supplies about 60% of the fatty acids and monoglycerides for the synthesis of TGs in the mammary gland; XOR plays a major role in the expression of lipid globules through the apical membrane of the mammocytes and is the second most abundant protein in the milk fat globule membrane (MFGM). As α-lactalbumin (α-La) modifies the specificity of UDP-galactosyltransferase (EC 2.4.1.22) in the synthesis of lactose and represents ~4% and ~40% of the protein in bovine and human milk, respectively, it is an enzyme modifier; it will not be discussed here, and the reader is referred to Chap. 8 for a comprehensive review.

The indigenous enzymes in milk arise from four principal sources:

- Blood plasma, through 'leaky junctions' between mammary cells.
- Secretory cell cytoplasm, some of which may be entrapped within some fat globules by the encircling MFGM during excretion from the cell.
- The MFGM itself, the outer layer of which is derived from the apical membrane of the mammary cell, and which, in turn, originates from the Golgi membranes; this is probably the source of most of the enzymes in milk.
- Somatic cells (leucocytes), which enter the mammary gland from the blood to fight bacterial infection (mastitis), and thereby enter milk.

Thus, most enzymes enter milk due to peculiarities of the mechanism by which milk constituents are excreted from the secretory cells. Milk does not contain substrates for many of the enzymes present, while others are inactive in milk due to unsuitable environmental conditions, e.g., pH or redox potential. However, many indigenous milk enzymes are significant from at least the following viewpoints:

- Deterioration, e.g., LPL (potentially, the most technologically significant enzyme in milk), proteinases, acid phosphatase and XOR.
- Indices of the thermal history of milk, e.g., alkaline phosphatase, lactoperoxidase (LPO), catalase, γ-glutamyl transferase, amylase and perhaps others.

This chapter is a modified and updated version of the reviews by Fox and Kelly (2006a, b).

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- Antimicrobial activity, e.g., lysozyme, XOR and LPO (which is exploited as a component of the LPO-H_2O_2-thiocyanate system for the cold pasteurisation of milk).
- Indices of mastitic infection, e.g., *N*-acetyl-β-D-glucosaminidase, catalase and acid phosphatase.
- Preservation of milk quality, e.g., sulphydryl oxidase (SHOx), superoxide dismutase (SOD).
- As a commercial source of enzymes, e.g., ribonuclease and LPO.
- Physiological functions in the neonate or in the mammary gland, e.g., bile salts-stimulated lipase and amylase, which are important in digestion by the human baby, and lysozyme, XOR and LPO, which have antimicrobial activity. However, these functions are not essential since the neonate can survive and thrive on heated milk in which these enzymes have been inactivated or on artificial formulae from which they are lacking.

Since the indigenous milk enzymes have no essential beneficial effect on the nutritional or organoleptic attributes of milk, their destruction by heat is one of the objectives of many dairy processes.

The first report of an enzyme, (lacto)peroxidase, in milk was by Arnold (1881), followed by reports of the presence of diastase (amylase) in the same decade. By 1902, the following enzymes had been reported in milk: peroxidase (oxidase), diastase (amylase), proteinase (galactase), 'fibrin ferment', lipase and 'salolase' (arylesterase) (Moro 1902). In 1902, Schardinger reported an enzyme in milk (now known as XOR) capable of oxidising aldehydes to acids but which also functions as a dehydrogenase. By the mid-1930s, the list of enzymes in milk was recognised as including proteinase, carbohydrase (amylase), esterases/lipases, peroxidase, xanthine oxidase (*aldehyd-katalase*) and catalase, with some reports of lactase (β-galactosidase) and salolase (arylesterase). By the 1950s, the list had been extended by the addition of alkaline phosphatase, lactase and coagulase (possibly thrombin). Probably reflecting the development of more sensitive assays, many new enzymes were detected in milk during the 1960s. For more detailed historical aspects of the discovery of these enzymes, the reader is referred to Fox and Kelly (2006a, b).

Andrews et al. (1992) reviewed the literature on 25 indigenous enzymes, and listed 34 other activities. Since 1992, the number of enzymes identified in milk has increased even further; Fox et al. (2003) described 32 enzymes and listed 37 other activities. It is likely that other enzymes, especially lysosomal enzymes, are present but have not been detected yet, perhaps due to the redox potential of milk, which is unsuitable for the action of some enzymes, or perhaps they have not even been assayed for. Barrett (1972) listed 53 enzymes that had been identified in lysosomes, only some of which have been reported in milk; it seems reasonable to assume that all lysosomal enzymes are present in milk. Multiple forms (isoenzymes) of many of the indigenous enzymes have also been reported.

Thus, the indigenous enzymes in bovine milk have attracted the attention of researchers for 130 years, and a very extensive literature has accumulated. In addition, the literature on the principal technologically significant enzymes has been reviewed individually (see the appropriate sections below).

There have been only occasional studies on the indigenous enzymes in the milk of other species, but some of the enzymes that have been studied show very marked differences from bovine milk. The indigenous enzymes in human milk also have been studied fairly extensively; human milk shows marked differences from bovine milk in the levels of several enzymes, e.g., a very high level of lysozyme, a bile salts-activated lipase (BSSL) in addition to the ubiquitous LPL, a high level of amylase but a low level of XOR and lacks LPO. Reviews include Hamosh (1988) and Hernell and Lonnerdal (1989). The indigenous enzymes in caprine and ovine milk have been reviewed by Moatsou (2010).

This article will review the literature on the principal indigenous enzymes in bovine milk and where possible in the milk of other species. These enzymes have been isolated and well characterised; they include the enzymes that were investigated during the early days of enzymology either because they were easily assayed or were technologically important. The minor enzymes are listed in Table 12.1; most of these have been identified

Table 12.1 Partial list of minor enzymes in milk, with associated EC numbers (modified from Farkye 2003)

Enzyme	EC Number
L-Iditol dehydrogenase	1.1.1.14
L-Lactate dehydrogenase	1.1.1.27
Malate dehydrogenase	1.1.1.37
Malic enzyme (oxaloacetate-decarboxylating) ($NADP^+$)	1.1.1.40
Isocitrate dehydrogenase ($NADP^+$)	1.1.1.42
Phosphogluconate dehydrogenase (decarboxylating)	1.1.1.44
Glucose-6-phosphate dehydrogenase	1.1.1.49
Amine oxidase	1.4.3.6
Polyamine oxidase	–
Fucosyltransferase	–
NADH dehydrogenase	1.6.99.3
Dihydrolipoamide dehydrogenase (diaphorase)	1.8.1.4
Lactose synthetase	2.4.1.22
Glycoprotein 4-β-galactosyltransferase	2.4.1.38
N-Acetyllactosamine synthase	2.4.1.90
CMP-*N*-acetylneuraminate-galactosyldiacylglycerol α-2,3-sialyltransferase	2.4.99.6
Thiamine-phosphate pyrophosphorylase	2.5.1.3
Aspartate aminotransferase	2.6.1.1
Alanine aminotransferase	2.6.1.2
RNA-directed DNA polymerase	2.7.7.49
Thiosulphate sulphurtransferase	2.8.1.1
Cholinesterase	3.1.1.8
Glucose-6-phosphatase	3.1.3.9
Phosphatidate phosphatase	3.1.3.4
Phosphodiesterase I	3.1.4.1
Arylsulphatase	3.1.6.1
β-Glucosidase	3.2.1.21
β-Galactosidase	3.1.1.23
α-Mannosidase	3.2.1.24
α-L-Fucosidase	3.2.1.51
Cytosol aminopeptidase (leucine aminopeptidase)	3.4.11.1
Cystyl-aminopeptidase (oxytocinase)	3.4.11.13
Trypsin	3.4.21.4
Inorganic pyrophosphatase	3.6.1.1
Adenosine triphosphatase	3.6.1.3
Thiamine pyrophosphatase (nucleoside diphosphatase)	3.6.1.6
Nucleotide pyrophosphatase	3.6.1.9
Fructose-bisphosphate aldolase	4.1.2.13
Carbonate dehydratase	4.2.1.1
Glucose-6-phosphate isomerase	5.3.1.9
Acetyl-CoA carboxylase	6.4.1.2

in milk only by their activity and have not been isolated from milk although similar enzymes have been isolated from other sources and, presumably, the enzymes in milk are generally similar. Some of these minor enzymes have been considered as indices of the heat treatment of milk.

12.2 Lactoperoxidase (EC 1.11.1.7)

Peroxidases, which are widely distributed in plant, animal and microbial tissues and secretions, catalyse the following reaction:

$$2HA + H_2O_2 \rightarrow A + 2H_2O$$

where HA is an oxidisable substrate or a hydrogen donor, which may be an aromatic amine, a (poly)phenol, an aromatic acid or a leuco dye. Many of these reducing agents are chromogenic, thus offering a method for detecting and quantifying peroxidase activity. The reducing substrates used most widely initially for the assay of LPO were guaiacol, pyrogallol and *p*-phenylenediamine, but 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] is now generally used, with measurement of A_{412}.

LPO was first demonstrated in milk by Arnold (1881), using *guajaktinctur* as reducing agent; he reported that the activity of LPO is lost on heating milk at 80°C. Louis Pasteur showed (1860–1864) that the spoilage of wine and beer could be prevented by heating at ~65°C for 30 min, and this process was first applied in 1891 to improve the quality of cream for buttermaking. As a means of controlling the spread of tuberculosis in cattle, legislation was introduced in Denmark in 1898 requiring that all skim milk returned by creameries to farmers should be flash (i.e. no holding period) pasteurised at 85°C (later changed to 80°C). Various tests were proposed to ensure that such milk was adequately pasteurised, but the most widely adopted was that developed by Storch (1898), who assayed LPO activity using *p*-phenylenediamine as reducing agent; the principle of the Storch test is still used to identify super-pasteurised milk, i.e. milk heated ≥76°C for 15 s. The original Storch test was purely qualitative, but

quantitative assays for LPO activity in milk were developed later. Because of the suitability of LPO as an indicator for super-pasteurised milk, its thermal denaturation has been intensively studied (e.g., Martin et al., 1990; Trujillo et al., 2007; Lorenzen et al., 2010).

The first study on the isolation of LPO was by Thurlow (1925), who obtained enriched preparations of LPO by fractional precipitation with $(NH_4)_2SO_4$. An improved method for the isolation of LPO from rennet whey by salting out, displacement chromatography and crystallisation was published by Polis and Shmukler (1953), who also characterised the enzyme. They reported that the enzyme is green in colour and that it was contaminated during the early stages of purification with a red protein, now called lactoferrin. LPO is a haem protein containing protoporphyrin IX with 0.069% Fe, a Soret band at 412 nm, an A_{412}:A_{280} ratio of 0.9, has a mass of 82 kDa and occurs as two isozymes, A and B.

During the following years, several methods for the isolation of LPO were published and knowledge on the characteristics of the enzyme was refined (e.g., Carlstrom 1969). Since LPO is cationic at the pH of milk, as are lactoferrin and some minor proteins, it can be easily isolated from milk or concentrated sweet (rennet) whey using a cationic exchange resin (e.g., Amberlite CG-50-NH_4) (Mitchell et al., 1994; Fweja et al., 2010) and further purified by a suitable technique, e.g., RP-HPLC (Carmen et al., 1990). The use of ultrasound-assisted ultrafiltration and aqueous two-phase extraction for recovery of LPO from whey was reported by Nandini and Rastogi (2011).

There are ten isozymes of LPO, arising from differences in the level of glycosylation and deamination of Gln or Asn. The mass of the enzyme is 78,030 Da, including sugars (8–10% of the mass of the enzyme) and the haem group. Carlstrom (1969) reported that LPO occurs as a homodimer but Sievers (1981), using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), found that the enzyme is a monomer. The primary structure of LPO was reported by Cals et al. (1991); it contains 612 amino acids and shows 55%, 54% and 45% identity with human myeloperoxidase, eosinophil peroxidase and thyroperoxidase, respectively. The LPO molecule is highly structured, with 65% β-structure, 23% α-helix and 12% unordered structure (Sievers 1980). A model of the tertiary structure of LPO, based on that of myeloperoxidase, was reported by de Wit and van Hooydonk (1996). LPO binds a Ca^{2+}, which has a major effect on its stability, including its heat stability. At pH below ~5.0, the Ca^{2+} is lost, with a consequent loss of stability.

LPO, which is synthesised in the mammary gland (Cals et al., 1991), was reported by Sievers (1980) and de Wit and van Hooydonk (1996) to be present also in human tears and saliva. Next to xanthine oxidase, LPO is the most abundant enzyme in milk, constituting ~0.5% of the total whey proteins (~0.1% of total protein; 30 mg/L). Hamosh (1988) reported that human milk is devoid of LPO but contains myeloperoxidase, which is generally similar to LPO, but Watanabe et al. (2000) reported a low level of LPO in human milk, about 5% of that in bovine milk. Apparently, human colostrum contains a high level of myeloperoxidase, derived from leucocytes, and a lower level of LPO. The level of myeloperoxidase decreases rapidly *post partum* and LPO is the principal peroxidase in mature human milk; it has been isolated and quantified by Shin et al. (2001). LPO has been suggested as a useful indicator of subclinical mastitis in goats (Seifu et al., 2007) but has been shown to be poorly suited to this purpose in the case of cows (Asadpour et al., 2008).

In the presence of low levels of H_2O_2 and SCN^-, LPO exhibits very potent bactericidal activity; this system is 50–100 times more effective than H_2O_2 alone. Most of the very extensive recent interest in LPO has focused on this aspect (see Björck 1992; Kussendrager and van Hooijdonk, 2000; Fox 2003; Pruitt 2003; Cankaya et al., 2010). Boulares et al. (2011a) showed significant increases in the refrigerated shelf life of raw ovine, bovine and caprine milk by activation of the LPO system through addition of sodium thiocyanate and sodium percarbonate. The impact of such treatments on the manufacture of cheese (Amornkul and Henning 2007; Boulares et al., 2011b) and yoghurt (Masud et al., 2010) has been studied. Boulares et al. (2011a, b) and Amornkul and Henning (2007) reported

alterations to manufacture and ripening of Saint-Paulin and Cheddar cheese, respectively, probably due to reduced growth of psychrotrophic bacteria in milk, following LPO treatment.

12.3 Catalase (EC 1.11.1.6)

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) catalyses the decomposition of H_2O_2, as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase also oxidises reducing agents, i.e. it has peroxidase activity. For a general review of catalases, see Wong and Whitaker (2003).

Catalase activity may be determined by quantifying the evolution of O_2 manometrically or by titrimetrically measuring the reduction of H_2O_2. Catalases are haem-containing enzymes that are distributed widely in plant, microbial and animal tissues and secretions; liver, erythrocytes and kidney are particularly rich sources. A catalase was among the first enzymes demonstrated in milk. Babcock and Russell (1897) reported that an extract of separator slime (somatic cells and other debris) could decompose H_2O_2, presumably indicating the presence of catalase.

Catalase activity in milk varies with feed, stage of lactation, and the level of activity increases markedly during mastitis (Johnson 1974). Catalase has been proposed as a useful indicator of mastitis (Kitchen 1981); however, it is now rarely used for this purpose; determination of somatic cell count (SCC), *N*-acetylglucosaminidase activity or electrical conductivity is used more frequently.

McMeekin and Polis (1949) reported that catalase is associated with casein but recent work indicates that it is concentrated in the cream and separator slime. According to Kitchen et al. (1970), 73% of the catalase in milk is in the skimmed milk, but the specific activity in the cream is 12-fold higher than that in skimmed milk; only about 8% of total catalase activity is in the ultracentrifugal casein pellet. Hence, the MFGM fraction is usually used as the starting material for the isolation of catalase from milk.

Considering that the level of catalase in milk is relatively high and that the enzyme is easily assayed, catalase was not isolated from milk until relatively recently. Various aspects of catalase in milk were reported by Prof. O. Ito in a series of papers during the period 1969–1983 (see Ito and Akuzawa 1983a), who isolated catalase from milk. The enzyme was purified 23,000-fold and crystallised and shown by gel permeation to have a molecular mass of 225 kDa. Ito and Akuzawa (1983b) reported that there were three isozymes in the catalase preparation isolated from cream. Ito and Akauzawa (1983c) reported that milk catalase was dissociated by SDS into five subunits ranging in molecular mass from 11 to 55 kDa. Bovine liver catalase is a homotetramer of 60–65 kDa subunits (total MW ~250 kDa). It seems likely that the structure of catalase in milk is similar to that enzyme and that the heterogeneity reported by Ito and Akuzawa (1983b) is due to proteolysis during isolation.

Catalase is relatively heat-labile (Farkye and Imafidon 1995; Hirvi et al., 1996) and was among the first indicators of pasteurisation investigated. More recently, the presence of active catalase has been considered as an indicator of cheese made from sub-pasteurised milk. There is general agreement that cheese made from raw milk ripens more quickly and develops a more intense (although not always a more desirable) flavour than cheese made from pasteurised milk (Fox et al., 2000). However, for public health reasons and in the interest of producing a consistent product, pasteurised milk is now generally used for cheesemaking. However, many varieties of cheeses are still made from raw milk, especially in southern Europe. Sub-pasteurised or thermised milk (e.g., that heated at 63–65°C for 16 s) has, in some cases, been considered as a compromise between raw and pasteurised milk for cheesemaking. The thermal inactivation of catalase was studied by Hirvi et al. (1996), and the possibility of using its inactivation as an index of thermised milk was investigated by Hirvi and Griffiths (1998). Although the inactivation of catalase was found to be a useful index of thermisation of milk (it being almost completely inactivated by heating at 65°C for 16 s), it was not suitable as an

index of cheese made from thermised milk owing to the production of catalase in the cheese during ripening, especially by coryneform bacteria and yeasts, if present

12.4 Xanthine Oxidoreductase [EC 1.1.3.22, 1.1.1.204]

In 1902, F. Schardinger showed that milk contains an enzyme capable of oxidising aldehydes to acids, accompanied by the reduction of methylene blue; this enzyme was then commonly called the 'Schardinger enzyme'. Morgan et al. (1922) showed that milk contains an enzyme capable of oxidising xanthine and hypoxanthine to uric acid, with the concomitant reduction of O_2 to H_2O_2; this enzyme was called xanthine oxidase (XO). Booth (1938) presented strong evidence that the Schardinger enzyme was, in fact, XO and partially purified it. Corran et al. (1939) isolated XO from whole milk and characterised its molecular properties and enzymatic activity. Ball (1939) showed that XO is concentrated in the cream phase of milk, from which they obtained highly purified preparations and showed that it requires FAD^+ for catalytic activity. XO can dehydrogenate xanthine under certain circumstances and is now usually called XOR.

An improved method for the isolation of XOR was published by Avis et al. (1955a) and the enzyme was crystallised and characterised by Avis et al. (1955a, b, c). XOR is concentrated in the MFGM, in which it is the second most abundant protein, after butyrophilin; it represents ~20% of the protein of the MFGM (~0.2% of total milk protein; ~120 mg/L). Isolation methods generally use washed cream as the starting material which is churned to yield a crude MFGM preparation. The early isolation methods used a proteinase (pancreatin) to solubilise XOR, but this causes limited proteolysis and changes the enzyme from a xanthine dehydrogenase (XDH) to an oxidase. Waud et al. (1975) used butanol to solubilise XO in milk followed by precipitation with $(NH_4)_2SO_4$ and chromatography on DEAE cellulose to isolate the enzyme; the effects of pancreatin on the properties of the enzyme were reported. Waud and Rajagopalan (1975, 1976) studied the interconversion of XO and XDH. Mangino and Brunner (1977) used deoxycholate to dissociate XOR from membrane lipoproteins and chromatography on hydroxylapatite to purify the enzyme. Plasmin, the principal indigenous proteinase in milk, was shown to hydrolyse XOR during isolation and explained the different values for the molecular mass of XOR reported by various authors. However, plasmin caused little hydrolysis of XOR in comparison with trypsin, chymotrypsin or papain (Cheng et al., 1988) and was considered to have little effect on XOR during isolation.

Silanikove and Shapiro (2007) reported that 33% of XOR activity in bovine milk is on the inner face of the MFGM, 20% on the inner face of skim milk membranes (which originate from the MFGM) and 47% is 'effectively soluble'. It was suggested by Silanikove et al. (2007) that the XOR on the inner membrane plays a non-enzymatic role in the expression of fat globules from the mammocytes while the extra-membranous XOR plays a role in the immune system of the mammary gland. However, Sharma et al. (2009) reported that only 5–10% of XO activity in bovine, buffalo and caprine milk is in the skim milk phase.

Because bovine milk is a very rich source of XOR, from which it is isolated relatively easily and because of its important and varied functions, XOR is a very well-characterised enzyme; currently it is probably the most studied of the indigenous milk enzymes. The extensive literature on XOR has been reviewed by Booth (1938), Whitney (1958), Fox and Morrissey (1981), Kitchen (1985), Massey and Harris (1997), Farkye (1992, 2003), Harrison (2004, 2006) and Fox and Kelly (2006a). XOR is a dimer of identical 146 kDa subunits, each containing ~1,330 amino acid residues. The gene for the enzyme from several sources has been cloned and shows a high degree of sequence conservation. Each XOR monomer contains 1 atom of Mo, 1 molecule of FAD^+ and 2 Fe_2S_2 redox centres. NADH acts as a reducing agent and the oxidation products are H_2O_2 and O_2. The milk of cows deficient in Mo has low XOR activity. Xanthine oxidase (XO; EC 1.1.3.22) and XDH (1.1.1.204) can be

interconverted by sulphydryl reagents, and XDH can be converted irreversibly to XO by specific proteolysis, e.g., by plasmin. The quaternary structure of XDH and XO was described by Enroth et al. (2000). Milk is a very good source of XOR, at least part of which is transported to the mammary gland *via* the blood stream. A similar enzyme is found in various animal tissues and in several bacterial species.

Early investigators reported that human milk lacks XOR, but Bradley and Gunther (1960), using a more sensitive assay, showed that human milk does contain XOR and that its level varies markedly during lactation. The XOR activity in human milk is low because 95–98% of the enzyme molecules lack Mo (Godber et al., 1997, 2005). Although XOR is a major protein in caprine MFGM (Cebo et al., 2010), the level of XOR activity in caprine and ovine milk is low (Atmani et al., 2004; Benboubetra et al., 2004; Gonzalez-Ronquillo et al., 2010). The level of XOR activity in human, ovine and caprine milk can be increased by supplementing the diet with Mo. A low level of XOR activity has been reported in camel milk (Al-Seeni 2009). XOR was identified in equine MFGM by Barello et al. (2008) but the level of activity was not reported.

12.4.1 Assay Methods

Xanthine oxidase activity can be assayed manometrically (uptake of O_2), potentiometrically, using a platinum electrode, polarographically or spectrophotometrically; the latter may involve the reduction of colourless triphenyltetrazolium chloride to a red product or the conversion of xanthine to uric acid which is quantified by measuring absorbance at 290 nm (see Fox and Kelly 2006a). XDH activity can be assayed by changes in NADPH concentration by absorbance at 290 nm.

12.4.2 Effect of Processing on XOR Activity in Milk

XOR activity in bovine milk varies substantially (Griffiths 1986). Processing treatments that damage or alter the MFGM affect the XOR activity in milk (Fox and Kelly 2006a). Measured activity is increased by storage at 4°C, heating at 70°C or by homogenisation. These treatments cause the release of XOR from the MFGM into the skim milk phase, rendering the enzyme more active. The heat stability and catalytic activity (Briley and Eisenthal 1974) of XOR are very dependent on whether it is a component of the MFGM or is dispersed in the aqueous phase of milk. Cold storage and homogenisation reduce the heat stability of XOR and explain the inconsistency of early work in which the history of the sample was unknown or unrecorded. XOR is most heat-stable in cream and least stable in skim milk. Homogenisation of concentrated milk prepared from heated (e.g., 90.5°C for 15 s) milk partially reactivates XOR, which persists on drying the concentrate; no reactivation occurs following more severe heating (e.g., 93°C for 15 s). Apparently, homogenisation releases potentially active, undenatured XOR from the MFGM. All the major milk proteins can act as either activators or inhibitors of XOR, depending on their concentration, and may have some significance in the activation, inactivation and reactivation of the enzyme (Hwang et al., 1967). Studies on the heat stability of XOR have been reviewed by Griffiths (1986), who investigated its stability in a pilot-scale high-temperature-short-time (HTST) pasteuriser; the enzyme was not completely inactivated by heating at 80°C for 120 s and a *Z*-value of 6.8°C was calculated.

12.4.3 Significance of Xanthine Oxidoreductase in Milk and Dairy Products

XOR has many functions in milk and dairy products:

- *As an index of heat treatment*: Andrews et al. (1987) suggested that XOR is a suitable indicator of milk heated in the temperature range 80–90°C, but Griffiths (1986) considered the natural variability in the level of XOR activity in milk to be too high for its use as a reliable index of heat treatment. Sharma et al. (2009)

suggested that XOR may be a suitable marker of heat treatment sufficient to kill *M. avium* ssp. *paratuberculosis*.

- *Lipid oxidation*: XOR can excite stable triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2$), a potent pro-oxidant. Some milk samples from individual cows, which undergo oxidative rancidity spontaneously (i.e. without contamination with metals or exposure to light), contain about 10× the normal level of XOR, and oxidation can be induced in normal milk by the addition of XOR to ~4× the normal level (Aurand et al., 1967, 1977). Heat-denatured or FAD-free enzyme is not a pro-oxidant.
- *Atherosclerosis*: It has been suggested that XOR enters the vascular system from homogenised milk and may be involved in atherosclerosis *via* oxidation of plasmalogens in cell membranes; this aspect of XOR attracted considerable attention in the early 1970s but the hypothesis has been discounted (see Clifford et al., 1983; Deeth 1983; Harrison 2002).
- *Reduction of nitrate in cheese*: Sodium nitrate is added to milk for many cheese varieties to prevent the growth of *Clostridium tyrobutyricum*, which causes flavour defects and late gas blowing in these cheeses; XOR reduces nitrate to nitrite, which is bactericidal, and then to NO.
- *Bactericidal activity*: XOR has strong antibacterial activity in the human intestine, and probably in the mammary gland *via* the production of peroxynitrite ($ONOO^-$) (Stevens et al., 2000; Godber et al., 2000; Atmani et al., 2005). XOR activity may contribute to the lower level of gastrointestinal infection in breast-fed babies compared to those bottle-fed. Indigenous XOR is inactivated in the production of infant formulae and the supplementation of such formulae with exogenous XOR (e.g., MFGM) has been proposed (see Harrison 2006). XOR, along with lysozyme and lactoferrin, is part of the innate immune system which evolved prior to the evolution of antibodies and is frequently referred as a housekeeping molecule (Vorbach et al., 2003).
- *Secretion of milk fat globules*: Probably the most important role of XOR in milk is now considered to be in the secretion of milk fat globules from the mammary secretory cells. The triglycerides in milk are synthesised in the endoplasmic reticulum (ER), where the TGs are formed into micro-lipid droplets and released through the involvement of a protein, acidophilin, which surrounds the globules. The ADPH-covered globules move towards the apical membrane of the cell, probably through a microtubular/microfilament system, and acquire additional coat material, cytoplasmic proteins and phospholipids. At the apical membrane, ADPH forms a disulphide-linked complex with two other proteins, butyrophilin, a transmembrane protein in the apical membrane of the cell, and dimeric XOR. Somehow, XOR causes blebbing of the fat globule through the membrane, and it is eventually pinched off and released into the alveolar lumen (McManaman et al., 2002, 2007; Vorbach et al., 2002). In the secretion of milk fat globules, XOR does not function as an enzyme. It is proposed that the secretion of milk fat globules is controlled by butyrophilin (Robenek et al., 2006).
- *Evolution of mammals*: Since XOR is a component of the innate immune system, it must have existed from a very early stage in evolution. It has been suggested (Vorbach et al., 2002, 2003, 2006) that the evolution of mammary glands (and hence mammals) was made possible through the function of XOR in the excretion of fat globules from the mammocytes. A second component of the innate immune system, lysozyme, evolved to become α-lactalbumin, the regulator of lactose synthesis. Thus, the production of the two principal sources of energy in milk, lipids and lactose, is possible through the involvement of two major components of the innate immune system. It is argued that the nutritional value of milk evolved subsequently to its immunological function and that the mammary gland evolved as a mucus skin gland, potentially with the objective of protecting the newly evolving mammalian skin from infectious diseases or to protect the surface of soft-shelled eggs or the newborn against dehydration and infection.

It is assumed that the newborn licked some secretion from the sebaceous glands and thus inadvertently obtained nutritional benefit. Various aspects of the origin and structure of the MFGM have been described by Aoki (2006) and Keenan and Mather (2006).

12.5 Superoxide Dismutase (EC 1.15.1.1)

SOD scavenges superoxide radicals, $O_2^{-\cdot}$ according to the reaction:

$$2\ O_2^{-\cdot} + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

The H_2O_2 formed may be reduced to $H_2O + O_2$ by catalase, peroxidase or a suitable reducing agent. The biological function of SOD is to protect tissue against free radicals of oxygen in anaerobic systems. Although oxygen radical-scavenging proteins had been isolated from cells previously, the significance of these proteins was not recognised until the work of J.M. McCord and I. Fridovich, in 1968–1969, which showed that the scavenging protein was an enzyme, which they called superoxidase dismutase. Since then, SOD has been identified in many animal and bacterial cells; the work has been reviewed by Fridovich (1975), McCord and Fridovich (1977) and Hara et al. (2003).

There are four isoforms of SOD, Cu/Zn-SOD, extracellular (EC) SOD, Mn-SOD and Fe-SOD. Cu/Zn-SOD is the most common form in mammals and has been isolated from a number of tissues, including bovine erythrocytes. It is a blue-green protein due to the presence of Cu (1 atom per monomer), removal of which by EDTA results in the loss of activity, which is restored by adding Cu^{2+}; it also contains 1 atom of Zn per monomer, which appears not to be involved in catalysis. The enzyme, which is very stable in 9 M urea at neutral pH, consists of two identical subunits of molecular weight 16 kDa (153 amino acid residues), linked by one or more disulphide bonds. The SOD in bovine milk is a Cu-Zn enzyme but human colostrum and mature milk contain both Cu-Zn and Mn types, the latter being eight and two times higher in human colostrum and milk, respectively (Kiyosawa et al., 1993). The amino acid sequence of Cu/Zn-SOD from several species has been reported (see Hara et al., 2003). The tertiary structure of Cu-Zn SOD from bovine erythrocytes was reported by Tainer et al. (1982). Mn-SOD and EC-SOD are tetrameric enzymes of 20 and 35 kDa subunits, respectively.

Bovine milk contains a low level of SOD (150 times less than in blood), which is present exclusively in the skim milk fraction; the SOD activity in bovine milk varies between animals and breeds (Holbrook and Hicks 1978; Granelli et al., 1995). The SOD in milk appears to be identical to the bovine erythrocyte enzyme (Hill 1975; Hicks et al., 1975; Keen et al., 1980). Assay methods for SOD are described by Stauffer (1989), Granelli et al. (1995) and Hara et al. (2003).

12.5.1 Significance

SOD inhibits lipid oxidation in model systems. The level of SOD in milk parallels that of XOR (but at a lower level), suggesting that SOD may offset the effect of the pro-oxidant XOR. Attempts to correlate the stability of milk to oxidative rancidity with indigenous SOD have been equivocal (Holbrook and Hicks 1978). Milk contains several pro- and antioxidants, the precise balance of which, rather than any single factor, determines oxidative stability (see Hicks 1980; Lindmark-Mansson and Akesson 2000).

SOD is more heat-stable in milk than in purified preparations. In milk it is stable to heating at 71°C for 30 min but it loses activity rapidly at slightly higher temperatures (Hicks 1980). Therefore, slight variations in pasteurisation temperature are critical to the survival of SOD in heated milk products and may contribute to variations in the stability of milk to oxidative rancidity. Homogenisation has little effect on the distribution of SOD in milk.

The possibility of using exogenous SOD to retard or inhibit lipid oxidation in dairy products has been considered. A marked improvement in the oxidative stability of milk was achieved by adding a low level of SOD (Aurand et al., 1977).

However, SOD is too expensive in comparison with chemical antioxidants for commercial use.

12.6 Sulphydryl Oxidase (EC 1.8.3-)

Gould (1940) reported that glutathione (GSH) added to raw or low-temperature heated milk disappeared quickly but was stable in milk that had been heated ≥80°C, suggesting that an enzyme was responsible for the destruction of GSH. An enzyme capable of oxidising the sulphydryl group of cysteine, GSH and proteins to disulphide bonds according to the following reaction:

$$2RSH + O_2 \rightarrow RSSR + H_2O_2$$

was first detected in milk by Kiermeier and Petz (1967) and purified by Janolino and Swaisgood (1975, 1978). The above reaction can also be catalysed by glutathione oxidase (GSHOx, EC 1.8.3.3) and γ-glutamyltransferase (GGT). Schmelzer et al. (1984) reported that antibodies raised against milk SHOx can immunoprecipitate GSHOx but not GGT. SHOx differs from thiol oxidase (EC 1.8.3.2) which requires FAD, and microbial SHOx. SHOx is widely distributed in cell membranes, including those of the mammary gland, kidney and pancreas but was not found in intestine, brain, heart, liver, lung, spleen or thymus (Clare et al., 1984). SHOx has been found in bovine, caprine, porcine, human and rat milk; Ouchterlony immunodiffusion showed that the enzyme from bovine and caprine milk and bovine kidney cross-reacted but the enzyme from human milk did not (Clare et al., 1984). There are several widely distributed enzymes called SHOx that oxidise sulphydryl groups in small molecules and/or proteins. Many of these enzymes found in mammalian tissues require FAD as a cofactor (see Tury et al., 2006), as does the SHOx of egg white (Hoober et al., 1996). An SHOx from *Aspergillus niger* does not require FAD (Vignaud et al., 2002); the properties of this enzyme are quite different from those of SHOx isolated from milk in terms of substrate specificity, molecular mass and isoelectric point (Janalino and Swaisgood 1992).

Milk SHOx is a glycoprotein (~10% carbohydrate) containing ~0.5 atoms of Fe per monomer (89 kDa) and does not require FAD. It has a strong tendency to associate, which makes it easy to isolate from whey by permeation chromatography on agarose or porous glass. A relatively simple, reproducible method for quantification of SHOx from milk involving covalent chromatography on cysteinylsuccinamidopropyl glass was published by Sliwkowski et al. (1983). The enzyme is optimally active at ~pH 7 and 35°C and is inhibited by metal chelators and sulphydryl-blocking reagents (Swaisgood and Janolino 2003). The kinetics of the enzyme were described by Sliwkowski et al. (1984).

SHOx oxidises reduced RNase and restores enzymatic activity, suggesting that its physiological function is the formation of specific disulphide bonds during the post-synthesis processing of proteins. It can convert XDH to xanthine oxidase (XO) by oxidising a sulphydryl group in the former (Clare et al., 1981; Blakistone et al., 1986). As discussed above, xanthine oxidase is involved in the expression of fat globules from the mammocytes and is a major protein in the MFGM; thus, it is possible that SHOx is involved in the expression of fat in the mammary gland.

The principal technological significance of SHOx in the dairy industry is in its ability to oxidise sulphydryl groups exposed and activated during high-temperature processing and which are responsible for the cooked flavour of such products. SHOx immobilised on glass beads reduces the cooked flavour of UHT-treated milk and remains active over a long period; this process has been patented (see Swaisgood and Janolino 2003) but has not been used commercially. Apparently, oxidation of the sulphydryl groups renders the product more stable to lipid oxidation, although sulphydryl groups *per se* are antioxidants.

SHOx activity is usually assayed on GSH at pH 7, by reacting with dithiodinitrobenzene, with which GSH forms a yellow product which is quantified by measuring absorbance at 412 nm (Janolino and Swaisgood 1975).

12.7 Glutathione Peroxidase (EC 1.11.1.9)

Glutathione peroxidase (GSHPOx) catalyses the reaction:

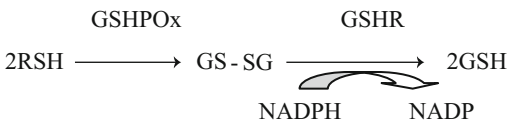
$$2GSH + ROOH \rightarrow GS\text{-}SG + ROH + H_2O$$

where GSH is glutathione (γ-Glu-Cys-Gly) and ROOH is a peroxide, including H_2O_2.

GSHPOx is widespread in the cytoplasm of animal tissues, especially erythrocytes from which it has been isolated. Its function is to protect the cell against the damaging effects of peroxides, as part of an antioxidative system which includes SOD. There are at least two forms of GSHPOx, cellular and extracellular (plasma) GSHPOx in mammals which are kinetically, structurally and antigenically distinct (see Douglas 1987; Avissar et al., 1991).

GSHPOx is a tetrameric protein of four identical subunits (21 kDa), each of which contains one atom of Se bound to a cysteine residue. The molecule has been well characterised, including elucidation of its primary, secondary and tertiary structures (see Epp et al., 1983; Liu and Luo 2003).

GSHPOx is assayed by a coupled reaction with glutathione reductase (GSHR; EC 1.6.4.2):



The loss of NADPH is quantified by measuring A_{340}. The nonenzymatic oxidation of NADPH is a problem (Chen et al., 2000). Alternatively, the decrease in the concentration of GSH can be quantified by reaction with dithiodinitrobenzoic acid or polarographically.

Milk contains a low level (27 ng/mL) of GSHPOx, more than 90% of which is the extracellular type. The level of GSHPOx in milk varies with the species (human> caprine> bovine) and diet (Debski et al., 1987). GSHPOx has no known enzymatic function in milk, in which it binds 30% of the total Se, an important trace element in the diet. Lindmark-Masson and Akesson (2001) reported the isolation of GSHPOx from bovine milk and developed an ELISA assay for the enzyme protein. Lindmark-Masson et al. (2001) reported that GSHPOx was stable to heating at 72°C for 2 min at the pH of milk and was stable for several days at 8°C. However, Stagsted (2006) claimed that the assay method used by the above and other authors measured superoxidase dismutase rather than GSHPOx and concluded that bovine milk does not contain GSHPOx activity.

A related enzyme GSHOx (EC 1.8.3.3), which catalyses the reaction:

$$2GSH + O_2 \rightarrow GSSG + H_2O_2$$

has not been reported in milk although antibodies raised against milk SHOx can immunoprecipitate GSHOx (Schmelzer et al., 1984).

12.8 γ-Glutamyl Transferase (EC 2.3.2.2)

γ-Glutamyl transferase (GGT) catalyses the transfer of γ-glutamyl residues from γ-glutamyl-containing peptides:

$$\gamma\text{-glutamyl-peptide} + X \rightarrow \text{peptide} + \gamma\text{-glutamyl-}X$$

where X is an amino acid.

GGT is associated with the membranes of a number of epithelial cells. Tate and Meister (1976) isolated GGT from rat kidney by affinity chromatography of a detergent extract of the tissue on concanavalin A. The enzyme is a glycoprotein and isoelectric focusing showed 12 isozymes, which differed in sialic acid content. SDS-PAGE showed that the enzyme is a dimer of subunits of molecular weight 46 and 22 kDa.

In milk, GGT is found in the membrane material in skim milk (~70%) or in the MFGM, from which it can be dissociated by detergents or organic solvents. The enzyme, which has been purified from the MFGM, has a molecular weight

of ~80 kDa and consists of two subunits of 57 and 25 kDa (determined by SDS-PAGE), both of which are glycoproteins (Baumrucker 1979, 1980). The enzyme, which associates strongly (Tate and Meister 1976; Kenny 1977), is optimally active at pH 8.5–9 and ~45°C and has an isoelectric point of 3.85. It is strongly inhibited by diisopropylfluorophosphate, iodoacetamide and metals, e.g., Cu^{2+} and Fe^{3+} (see Farkye 2003). GGT activity in human and bovine milk varies during lactation, being highest in colostrum; variation in its activity in buffalo milk over lactation was reported by Pero et al. (2006).

GGT functions in the regulation of cellular GSH and may be involved in the transport of amino acids from blood into the mammary gland *via* the so-called γ-glutamyl cycle (Meister 1973; Kenny 1977) and thus may be involved in the biosynthesis of milk proteins (Baumrucker and Pocius 1978).

GGT is usually assayed using γ-glutamyl-*p*-nitroanilide as substrate; the liberated *p*-NA can be determined by measuring the absorbance at 410 nm or by reaction with naphthylethylenediamine and measuring the absorbance at 540 nm (McKellar et al., 1991).

From a dairy technologist's viewpoint, GGT is of interest mainly because of its heat stability characteristics, as for many other enzymes. As discussed earlier, alkaline phosphatase is the test enzyme usually used to evaluate the effectiveness of HTST pasteurisation; however, as discussed, reactivation of AlP in UHT-treated products poses problems in the interpretation of the test. Based on a comparative study on the heat-stability characteristics of a number of indigenous enzymes in milk, Andrews et al. (1987) concluded that GGT is appropriate for monitoring heat treatments in the range of 70–80°C for 16 s. This conclusion has been confirmed in pilot-scale studies (Patel and Wilbey 1989; Carter et al., 1990). In whole or skim milk, GGT is completely inactivated by heating at 78°C for 15 s (Patel and Wilbey 1989) or 77°C for 16 s (McKellar et al., 1991). No reactivation was found under various conditions and little seasonal variation occurs. As little as 0.1% or 0.25% raw milk could be detected in pasteurised skim or whole milk, respectively (McKellar et al., 1991).

Linear models for the thermal inactivation of GGT and LPO in a HTST pasteuriser were developed by McKellar et al. (1996). The relationship between % inactivation and pasteurisation equivalent was more linear than the relationship for AlP, possibly due to the presence of more than one isozyme of AlP (McKellar et al., 1996). GGT was ~9 times more stable in ice cream mix than in whole milk (McKellar 1996). Thus, it appears that GGT is a suitable enzyme for estimating the intensity of heat treatment of milk in the range 72–77°C for 15 s; this was recently proposed for camel milk (Wernery et al., 2008). The pressure-resistance of GGT was reported by Pandey and Ramaswamy (2004).

GGT is absorbed from the gastrointestinal tract, resulting in high GGT activity in the blood serum of newborn animals fed colostrum or early-lactation breast milk. Since GGT is inactivated by the heat treatment to which infant formulae are subjected, the level of serum GGT activity in infants can be used to distinguish breast-fed from formula-fed infants (see Farkye 2003).

γ-Glutamyl peptides have been isolated from Comté (Roudot-Algaron et al., 1994) and Gouda and Blue (Toelstede and Hofmann 2009) cheese; since casein contains no γ-glutamyl bonds, the presence of these peptides in cheese may suggest GGT activity in cheese but there appear to be no data to support this hypothesis.

12.9 Lipases and Esterases

Lipase is, potentially, the most important indigenous enzyme technologically. Its activity causes hydrolytic rancidity, an off-flavour defect that has been recognised since the pre-industrialisation of dairying: Dunkley (1946) cited the description by Lawrence in 1726 of a bitter flavour in milk and cream, which Dunkley considered to be hydrolytic rancidity, caused by a lipase. Hydrolytic rancidity has been a major problem for many years, especially after the introduction of pipeline milking machines in the 1950s (see Herrington 1954). The influence of various factors on the development of rancidity was described by Dunkley (1946), Herrington (1954), Jensen

(1964), Downey (1975), Deeth and Fitz-Gerald (1976, 1995, 2006) and Deeth (2006).

In addition to causing off-flavours in milk and dairy products, lipolysis, by reducing the surface activity of milk, reduces its foaming capacity, e.g., in cappuccino coffee, and its whipping and churning time (Deeth and Fitz-Gerald 2006). However, milk lipase contributes positively to the flavour of raw-milk cheese (Collins et al., 2003; Deeth and Fitz-Gerald 2006).

The presence of lipolytic activity in milk was reported by Moro (1902) and this view was confirmed by several workers (see Palmer 1922; Corbin and Whittier 1965; Fox and Kelly 2006a). Palmer (1922), who critically reviewed the earlier studies on milk lipase and lipolysis in milk, concluded that most of the earlier studies were defective because of failure to include proper controls and/or because soluble esters were used as substrate; he found no evidence of lipolytic activity in normal bovine milk but Rice and Markley (1922) presented strong evidence indicating the presence of lipase in milk. Herrington (1954) compiled an extensive review on milk lipase but concentrated on various aspects of lipolysis, rather than on the enzyme, which at that stage had not been isolated and had been characterised only in general terms.

Tarassuk and Frankel (1957) claimed that there were at least two lipases in milk, 'plasma lipase' and 'membrane lipase'; the latter was considered to be responsible for spontaneous lipolysis (see Corbin and Whittier 1965). Gel permeation and ion-exchange chromatography indicated that there are several lipases in milk, but it seems likely that these multiple forms of lipase were due the self-association of lipase or to association with other milk proteins (Fox and Kelly 2006a). At least 90% of the lipase in milk is associated with the casein micelles (Gaffney et al., 1966), but it can be dissociated from the micelles by treatment with 1 M NaCl (Downey and Andrews 1966), dimethylformamide (Fox et al., 1967) or heparin (Hoynes and Downey 1973).

A very low molecular weight (~8 kDa) lipase was purified from separator slime by Chandan and Shahani (1963a, b). This lipase probably originated from somatic cells and it was considered to be only a minor lipase in milk (Castberg et al., 1975).

Quigley et al. (1958) and Korn (1962) reported that milk contains a lipoprotein lipase (LPL). A lipase was isolated from skimmed milk by Fox and Tarassuk (1968) and characterised by Patel et al. (1968). This enzyme was inhibited by organophosphates and had a molecular mass of 210 kDa (by gel permeation chromatography). Fox and Flynn (1980) showed that the lipase isolated from milk by Fox and Tarassuk (1968) is an LPL.

Brockerhoff and Jensen (1974) and Jensen and Pitas (1976) proposed that milk contains both lipase and LPL. LPL was isolated from milk by Egelrud and Olivecrona (1972); it was found to be a homodimer (molecular mass ~90 kDa) with each monomer containing 450 amino acid residues. It originates from the vascular endothelial surfaces, where it is bound by heparin sulphate chains and plays a very important role in the synthesis of lipids in the mammary gland (see McBride and Korn 1963; Liesman et al., 1988; Olivecrona et al., 2003; Palmquist 2006). Askew et al. (1970) and Castberg et al. (1975) showed that the LPL isolated from mammary tissue was the same as that isolated from milk.

LPL is strongly involved in the biosynthesis of milk lipids: all of the C18 acids and ~50% of the C16 acids in ruminant milk lipids are derived from dietary lipids which are transported to the mammary gland in chylomicrons, from which FAs are released by mammary LPL and incorporated into TGs in the mammocytes (see Barber et al., 1997 and Palmquist 2006).

Owing to its importance for lipid metabolism in animal tissue, including the absorption of dietary lipids, the biosynthesis of lipids, including milk lipids, and cardiovascular diseases (Goldberg 1996; Mead et al., 2002; Glisic et al., 2008), LPL is very well characterised (see Olivecrona and Bengtsson 1984; Senda et al., 1987; Olivecrona and Bengtsson-Olivecrona 1991; Auwerx et al., 1992; Wong and Schotz 2002; Olivecrona et al., 2003). LPL has been isolated from several tissues of several species (Cryer 1987). Milk is a rich source of LPL (~1 mg/L) from which it can be isolated relatively easily by affinity chromatography on heparin agarose.

The enzyme is a homodimer; each monomer of human LPL consists of 448 amino acid residues, with a molecular mass of 50,394 Da and containing 8% carbohydrate. The isoelectric point of the protein is 8.91; it contains 10 cysteine residues, all of which are in disulphide linkages and two *N*-glycosylation sites, Asn_{44} and Asn_{361} (Yang et al., 1989).

The secondary and tertiary structures of LPL have not been determined, but its 3D structure has been deduced by analogy with the structure of pancreatic lipase with which LPL has a high level of homology. LPL is a member of a family of lipases, which evolved from a common ancestral lipase; the principal members are pancreatic lipase (PL) which hydrolyses emulsified triglycerides, and two lipoprotein-metabolising lipases, LPL and hepatic lipase (HL) (see Borgstrom and Brockman 1984). The amino acid sequences of human LPL and human PL show 30% homology (van Tilbeurgh et al., 1994). PL requires colipase, a protein which contains 96 amino acid residues, which anchors the enzyme at the lipid/water interface (see Wong 2003), while LPL is activated by apolipoprotein CII, a small protein containing 79 amino acid residues. Hepatic lipase, which is produced in the liver, plays a major role in lipid metabolism; it is a glycoprotein of mass ~62 kDa and is activated by apolipoprotein AII but inhibited by apolipoproteins AI, CI, CII and CIII (Jahn et al., 1983). The literature on HL has been reviewed by Perret et al. (2002) and Jansen et al. (2002). The three lipases have Ser, Asp and His at the active site.

Pancreatic lipase, which is regarded as the typical lipase, is stimulated by bile salts, which serve as emulsifiers or to complex with, and remove, the liberated fatty acids, which inhibit the enzyme. Other molecules that bind or react with fatty acids, e.g., calcium salts, blood serum albumin or β-lactoglobulin, also stimulate PL. Pancreatic lipase is a well-characterised monomeric glycoprotein with a molecular mass of ~50 kDa (450 amino acid residues) and is optimally active at ~pH 9 (Van Tilbeurgh et al., 1992, 1994).

Reflecting its importance in the biosynthesis of milk fat and its role in hydrolytic rancidity, mammary/milk lipase/LPL has been the subject of several reviews, including those by Herrington (1954), Chandan and Shahani (1964), Shahani et al. (1973), Brockerhoff and Jensen (1974), Jensen and Pitas (1976), Olivecrona and Bengtsson (1984), Olivecrona and Bengtsson-Olivecrona (1991), Olivecrona et al. (1992, 2003) and Deeth and Fitz-Gerald (1995, 2006).

The pH and temperature optima of LPL are ~9°C and 37°C, respectively. Under optimum conditions, the k_{cat} of LPL is ~3,000 s^{-1} and milk contains sufficient lipase (1–2 mg/L; 10–20 nM) to cause hydrolytic rancidity in 10 s. However, in most milk samples, LPL causes hydrolytic rancidity only if the MFGM is damaged, e.g., by agitation, foaming, cooling/warming, freezing or homogenisation. The various factors that activate lipolysis cause a shift of LPL from the casein micelles to the cream phase (Cartier and Chilliard 1989; Cartier et al., 1989). The milk of some cows undergoes spontaneous lipolysis, i.e. without the need for an activation step. Initially, it was proposed that such milks contained a second (membrane) lipase (Tarassuk and Frankel 1957). However, it now appears that they contain either a high level of apolipoprotein CII, which activates LPL, or that normal milk has a higher level of proteose peptone eight, which inhibits LPL (see Deeth 2006).

According to de Foe et al. (1982), caprine milk contains only ~4% as much lipolytic activity as bovine milk and contains two LPL isozymes, both with a higher molecular mass than bovine LPL. The yield of LPL is increased markedly by chromatography on hydroxyapatite and heparin-Sepharose 4B, probably due to the removal of an inhibitor(s) (a much smaller increase in yield was obtained for ovine LPL). Badaoui et al. (2007) identified two polymorphisms in caprine LPL, one of which occurred in the signal peptide and which may affect the expression of the enzyme and which is breed-dependent.

Most (~80%) of the LPL in bovine milk is associated with the casein micelles, with <10% in the cream phase, but in caprine milk <10% of the LPL is associated with the micelles, with 45% each in the cream and serum phases. The differences in the distribution pattern of LPL may explain the greater susceptibility of caprine milk

to spontaneous lipolysis and the characteristic flavour of goat milk, which is due to minor branched-chain fatty acids, 4-methyl- and 4-ethyl-octanoic acids. The lipolytic system in caprine milk and its significance for various aspects of caprine milk were reviewed by Chilliard et al. (1984, 2003). Ovine milk contains only ~10% of the lipolytic activity of bovine milk (Chandan et al., 1968). Ovine LPL has been described by Edwards et al. (1993) and Bonnet et al. (2000).

Equine milk contains about the same level of lipolytic activity as bovine milk and is due to an LPL (Chillard and Doreau 1985) which has not been isolated or characterised. Guinea pig milk contains high LPL activity but rat milk has low activity (Hamosh and Scow 1971). Guinea pig LPL was purified by Wallinder et al. (1982) and found to be similar to the LPL of bovine milk.

12.9.1 Bile Salts-Stimulated Lipase (EC 3.1.1.3)

It has been known since the early years of the twentieth century that human milk has considerably higher lipolytic activity than bovine milk (see Palmer 1922). In fact, human milk, and that of some other species, contains a second lipase in addition to LPL, i.e. bile salts-stimulated lipase (BSSL) which is similar to the broad-specificity pancreatic carboxylic ester hydrolase (CEH; also called cholesterol ester hydrolase; see Chen et al., 1998). This enzyme was studied by E. Freudenberg during the period 1927–1953 (see Freudenberg 1953), but the significance of these studies was not generally appreciated at that time. BSSL is considered to be very important for the digestion of lipids by human babies who secrete low levels of both pancreatic lipase and bile salts (see Shahani et al., 1980; Hernell and Bläcksberg 1991). The significance of pre-duodenal lipases (lingual lipase, pre-gastric esterase and gastric lipase) in fat digestion by human infants was described by Hamosh (1990). Fatty acids released by either LPL or BSSL may have an antibacterial effect (see Hamosh 1988).

BSSL was isolated from human milk by Blackberg and Hernell (1981); it is synthesised in the mammary gland and represents ~1% of the total protein in human milk. The enzyme is inactivated by pasteurisation, as a result of which the absorption of lipids by preterm infants is reduced by ~30%. The gene for human BSSL was cloned by Nilsson et al. (1990), who compared its derived amino acid sequence with that of CEH. The sequence consists of 722 amino acid residues; it has a total molecular mass of ~105 kDa, including 15–20% carbohydrate (molecular mass of the polypeptide, 76,271 Da; Nilsson et al., 1990). BSSL shows high homology with lysophospholipase from rat pancreas and acetylcholine esterase, as well as to CEH. BSSL has been found only in the milk of higher primates. The structure of pancreatic CEH, a monomeric protein of ~65.5 kDa, has been described by Rudd and Brockman (1984) and Chen et al. (1998). The structure of the human CEH gene was reported by Kumar et al. (1992). There is quite an extensive literature on BSSL, which was reviewed by Olivecrona and Bengtsson (1984), Corry (2004) and Deeth and Fitz-Gerald (2006).

12.9.2 Phospholipase

Bulk herd milk was reported by Shukla and Tobias (1970) to possess significant phospholipase-D activity, which, it was suggested, might increase the resistance of milk to oxidative rancidity; however, Chen et al. (1978) failed to identify phospholipase-D in milk. O'Mahony and Shipe (1972) reported that phospholipase-C stabilised milk to oxidation.

12.9.3 Esterases

Esterases are distinguished from lipases by their preference for soluble rather than emulsified ester substrates. As discussed by Palmer (1922), early studies on milk lipase did not distinguish between lipases and esterases. Milk contains several esterases (Kitchen 1985), the most significant of which are arylesterases (EC 3.1.1.7), cholinesterase (EC 3.1.1.8) and carboxylesterase (3.1.1.1). Arylesterase (also called

solalase) was among the first enzymes reported in milk (see Moro 1902). It has been isolated from milk and characterised (Kitchen 1971). Arylesterase activity is high in colostrum and during mastitis but it probably has no technological significance (see Kitchen 1985).

12.10 Proteinases

Babcock and Russell (1897) extracted from separator slime a trypsin-like proteinase, which they called 'galactase' (derived from *gala*, Greek for milk; genative, *galaktos*). They proposed that this enzyme is involved in cheese ripening, and it seems likely that it originated from leucocytes (somatic cells), which are rich in cathepsins, and was not the principal indigenous milk proteinase, plasmin, which is associated with the casein micelles, rather than somatic cells. The milk of several species (cow, goat, sheep, horse, donkey, bison, pig and human) was subsequently shown to contain proteolytic activity, porcine milk being a particularly rich source (Babcock et al., 1898).

The presence of an indigenous proteinase in milk, mainly in separator slime, was confirmed by Tatcher and Dahlberg (1917). However, the presence of an indigenous proteinase in milk was doubted for many years, contaminating bacteria being considered as a possible source of the activity detected. Warner and Polis (1945) reported a low level of proteolytic activity in acid casein which caused a decrease in the viscosity of sodium caseinate during storage, with a concomitant increase in pH 4.6-soluble N. Using aseptically drawn, low-bacterial-count milk with added antibiotics, Harper et al. (1960) finally showed that milk does indeed contain an indigenous proteinase(s), although the authors considered the level to be so low as to be insignificant.

12.10.1 Plasmin (EC 3.4.21.7)

Milk is now known to contain several indigenous proteinases, the principal of which is plasmin (fibrinolysin). In fact, milk contains the complete plasmin system found in blood: plasmin, plasminogen, plasminogen activators (PAs) and inhibitors of both PAs and plasmin. This system enters milk from blood, and plasmin activity increases in situations where there is an increased influx of blood constituents into milk, i.e. during mastitic infection and in late lactation. Plasmin activity has been linked to the physiology of milk secretion in the udder (Silanikove et al., 2006); in particular, products of the hydrolysis of β-casein by plasmin (proteose peptone 8f, fragment β-casein f1-28) have been shown to be able to downregulate milk secretion in the udder.

In milk, plasminogen, plasmin and PAs are associated with the casein micelles and are concentrated in rennet-coagulated cheese curds and casein, while the inhibitors of PAs and plasmin are soluble in the milk serum (Ismail et al., 2006). During storage of milk, activation of plasminogen to plasmin can occur, even at refrigeration temperatures (Schroeder et al., 2008; Lu et al., 2009). Owing to changes in practices in the dairy industry, e.g., improved bacterial quality, extended storage on farms and factories and the introduction of high-temperature processed milk (plasmin is very heat-stable), plasmin has become a very significant enzyme in milk and, consequently, the subject of considerable research. The literature has been reviewed regularly, e.g., Humbert and Alais (1979), Grufferty and Fox (1988), Bastian and Brown (1996), Kelly and McSweeney (2003) and Ismail and Nielsen (2010).

Plasmin is a very well-characterised proteinase, as are the various components of the plasmin system (see Kelly and McSweeney 2003). Bovine plasminogen is a single-chain glycoprotein containing 786 amino acid residues, with a calculated molecular mass of 88,092 Da; the polypeptide exists as five disulphide-linked loops ('kringles'). Plasminogen is converted to plasmin by cleavage of the Arg_{557}-Ile_{558} bond by specific proteinases, of which there are two types, urokinase-type and tissue-type PA. The impact of heating milk on the distribution of PA and activation of plasminogen was studied by Burbrink and Hayes (2006), Prado et al. (2006) and Wang et al. (2007). Plasmin is optimally active at pH 7.5 and 37°C; it is quite heat-stable

and partially survives UHT processing and other high-temperature processes (Newstead et al., 2006; van Asselt et al., 2008). Recent studies have examined the effect of new processing technologies on plasmin, including ultra-high-pressure homogenisation (Iucci et al., 2008), high-pressure treatment (Hurpertz et al., 2004; Moatsou et al., 2008a) and microfiltration (Aaltonen and Ollikainen 2011).

Plasmin is highly specific for peptide bonds containing Lys or Arg at the N-terminal side. The specificity of plasmin on α_{s1}-, α_{s2}- and β-caseins in solution has been determined (see Kelly and McSweeney 2003); it has little or no activity on κ-casein (CN), β-lg or α-la (in fact, denatured β-lg is an inhibitor; Grufferty and Fox 1986). In milk, the principal substrate for plasmin is β-CN, from which it produces γ^1- (β-CN f29-209), γ^2- (β-CN f106-209) and γ^3- (β-CN f108-209) caseins and proteose peptone (PP)5 (β-CN f1-105/107), $PP8_{slow}$ (β-CN f29-105/107) and $PP8_{fast}$ (β-CN f1-29).

Long et al. (1958) isolated a proteinaceous fraction, which they called λ-caseins, by ultracentrifugation of a crude κ-casein preparation. El-Negoumy (1973) prepared this fraction from milk by ammonium sulphate precipitation in the presence of *N*,*N*-dimethyl formamide and, by electrophoretic and chromatographic techniques, identified at least nine components with a higher electrophoretic mobility than α_{S1}-casein. Aimutis and Eigel (1982) concluded that many of the peptides in this fraction are produced from α_{S1}-casein by plasmin. O'Flaherty (1997) studied the λ-casein fraction of milk and identified several peptides that could have been produced from α_{s1}-casein by either plasmin or cathepsin D.

Plasmin contributes to primary proteolysis in cheese, most significantly in high-cooked varieties in which the coagulant is extensively denatured (Sheehan et al., 2007); it may cause age gelation of UHT-sterilised milk (although this has not been proven unequivocally; Newstead et al., 2006; Gaucher et al., 2009), can affect the coagulation properties of milk (Srinivasan and Lucey 2002) and may reduce the yield of cheese and casein owing to the loss of proteose peptones in whey (Mara et al., 1998). Cheesemaking parameters such as salting and cooking temperature can influence plasmin activity in cheese, and hence the contribution of the enzyme to ripening (Choi et al., 2006); increasing the temperature at which curds are cooked increases plasmin activity in the cheese, and the concomitant increased inactivation of chymosin further increases the relative contribution of plasmin to primary proteolysis of the caseins.

12.10.2 Cathepsin D (EC 3.4.23.5)

The second proteinase identified in milk was cathepsin D (Kaminogawa and Yamauchi 1972), which originates from lysosomes but is present in acid whey (Larsen et al., 1996). As with plasmin, cathepsin D is part of a complex system, including inactive precursors (for review, see Hurley et al., 2000). The major form of cathepsin D in milk is the inactive zymogen, procathepsin D, although milk also contains low levels of the mature form of the enzyme. The level of cathepsin D in milk is correlated significantly with SCC (O'Driscoll et al., 1999), although it is not clear whether this reflects increased production of cathepsin D and/or increased activation of precursors (see Hurley et al., 2000).

Kaminogawa et al. (1980) and McSweeney et al. (1995) showed that partially purified cathepsin D from milk hydrolysed α_{s1}-casein to a peptide with the same molecular mass or electrophoretic mobility as α_{s1}-CN (f24-199), which is one of the primary peptides produced from α_{s1}-casein by chymosin. The proteolytic specificity of cathepsin D on β-casein is also similar to that of chymosin. Cathepsin D can cleave κ-casein but has very poor milk clotting properties (McSweeney et al., 1995; Larsen et al., 1996). Two cleavage sites of cathepsin D on α-lactalbumin have been identified, but native β-lactoglobulin is resistant to cleavage by this enzyme (Larsen et al., 1996). Hayes et al. (2001) and Moatsou et al. (2008a,b) reported on the heat and pressure resistance of cathepsin D in milk, and the former concluded that some cathepsin D activity could survive heat treatments such as HTST pasteurisation and the cooking applied during the manufacture of Swiss-type cheese.

12.10.3 Other Proteinases

Somatic cells contain several other proteinases, including cathepsins B (EC 3.4.22.1), L (EC 3.4.22.15) and G (EC 3.4.21.20), and elastase (EC 3.4.21.36), which have received limited attention to date (see Kelly and McSweeney 2003). The lysosomal cysteine proteinases were reviewed by Kirschke et al.(1998); in addition to cathepsins B, L and G, these include cathepsins S (EC 3.4.22.27), K (EC 3.4.22.38), T (EC 3.4.22.24), N and O, dipeptidyl peptidase I (EC 3.4.14.1) and legumain (EC 3.4.22.34) (in legumes). Magboul et al. (2001) presented evidence for the presence of cysteine protease activity, most likely that of cathepsin B (based on immunological analysis), in milk; the specificity of this enzyme on the caseins, which is very broad and shared some bond preferences with both plasmin and chymosin, was determined by Considine et al. (2004).

Presumably, most of these enzymes are present in milk but are inactive owing to the high redox potential of milk, under which conditions the active-site sulphydryl group would be oxidised; the authors do not know if attempts have been made to assay these enzymes under reducing conditions.

12.10.4 Relative Significance of Proteinases in Milk

The relative significance of the different proteinases in milk to hydrolysis of proteins clearly depends on a number of factors, in particular the health status of the cow. In milk from a healthy cow, the predominant activity is that of plasmin, but as the SCC increases, the relative importance of lysosomal proteinases increases. A number of other recent studies have teased out the complex system of proteolytic enzymes in high SCC milk (Le Roux et al., 2003; Somers et al., 2003; Larsen et al., 2004, 2006, 2010a; Haddadi et al., 2006; Wedholm et al., 2008; Albenzio et al., 2009; Santillo et al., 2009). These studies have collectively suggested strong evidence of the activity of elastase, cathepsin B and several other lysosomal enzymes in milk with either naturally or artificially high SCC; a number of recent studies have used bacteria-free systems where mastitis is induced by infusion of a bacterial antigen such as lipopolysaccharide or lipoteichoic acid, which allows the enzymology of the resulting milk to be studied in the absence of confounding bacterial activities. Using such an approach, Larsen et al. (2010b) found evidence of amino- and carboxypeptidase activity in high SCC milk, perhaps originating from cathepsin H. The specific contribution of lysosomal proteases from polymorphonuclear leucocytes (PMN), the main type of somatic cell recruited during mastitic infection, has been elucidated by Le Roux et al. (2003) and Haddadi et al. (2006).

12.10.5 Proteinases in Human Milk

Greater proteolytic activity in human than in bovine milk was reported by Storrs and Hull (1956), Hernell and Lonnerdal (1989) and Heegaard et al. (1997). However, Korycka-Dahl et al. (1983) reported that the level of plasmin is about the same in human and bovine milk but that the former contains about four times more plasminogen. Ferranti et al. (2004) identified several casein-derived peptides, some of which may be biologically active, in human milk. Their results suggest that human milk contains several proteinases and peptidases in addition to plasmin, including amino- and carboxypeptidases. The mechanisms of proteolysis in human milk were further studied in detail, in particular in the context of milk from mothers giving birth prematurely, by Armaforte et al. (2010); the milk from mothers of premature infants was found to have higher plasmin activity and consequently greater proteolysis of casein than that of term mothers, which may be to provide more vulnerable newborn infants with a higher level of easily digested proteins and peptides, or result from the mammary gland producing milk under stressed conditions.

12.11 Alkaline Phosphatase (EC 3.1.3.1)

12.11.1 Introduction

Milk contains several phosphatases, the principal ones being alkaline and acid phosphomonoesterases, which are of technological significance. Milk

also contains ribonuclease, which has no known function or significance in milk, although it may be significant in the mammary gland. The alkaline and acid phosphomonoesterases in milk have been studied extensively; the literature has been reviewed by Fox and Morrissey (1981), Kitchen (1985), Andrews et al. (1992), Shakeel-Ur-Rehman et al. (2003), Fox (2003) and Fox and Kelly (2006b).

The occurrence of a phosphatase in milk was first recognised in 1925 by F. Demuth (see Whitney 1958). Subsequently characterised as an alkaline phosphatase indigenous to milk (Graham and Kay 1933), it became significant when it was shown that the time-temperature combinations required for the thermal inactivation of alkaline phosphatase were slightly more severe than those required to kill *Mycobacterium tuberculosis*, then the target microorganism for pasteurisation (Kay and Graham 1933). The enzyme is readily assayed, and a test procedure based on the inactivation of AlP was developed as a routine quality control test for HTST pasteurisation of milk (Kay and Graham 1935).

The AlP activity of bovine milk varies considerably between individuals and herds, and throughout lactation (minimum at ~1 week and maximum at ~28 weeks); activity varies inversely with milk yield but is independent of fat content, breed and feed (Haab and Smith 1956). The variability in AlP activity in human milk was described by Stewart et al. (1958).

12.11.2 Isolation and Characterisation

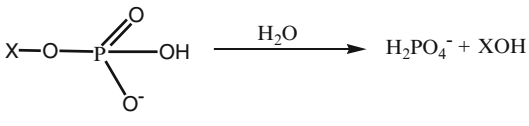
Kay and Graham (1933) observed that AlP is concentrated in cream and released into buttermilk on churning (in fact about 50% of AlP is in the skimmed milk but the specific activity is higher in cream). Zittle and DellaMonica (1952) partially purified AlP from whey, and Morton (1950) showed that lipoprotein particles, which he called 'microsomes' (Morton 1953), are a rich source of AlP and many other indigenous enzymes (Morton 1953; Zittle et al., 1956). AlP can be released from the microsomes by treatment with *n*-butanol (Zittle and DellaMonica 1952; Morton 1953) which, combined with salting-out and ion-exchange or gel permeation chromatography, formed the basis of all early methods for the isolation of AlP from milk (Zittle and DellaMonica 1952; Morton 1953, 1954; Gammack and Gupta 1967; Le Franc and Han 1967; Buruiana and Marin 1969; Linden et al., 1974). Chromatography of *n*-butanol extracts of MFGM on Concanavalin A Agarose/Sepharose 4B/Sephacryl S-200 has been used in a number of methods developed recently for the isolation of AlP from milk (Vega-Warner et al., 1999; see Shakeel-ur-Rehman et al., 2003). Bingham and Malin (1992) reported that AlP is released from the phospholipids of the MFGM by treatment of milk with phosphatidylinositol-specific phospholipase C, indicating that AlP is bound to the mammary cell membranes and the MFGM *via* phosphatidylinositol. This is the common form of linkage of AlP to membranes (see Moss 1992).

AlP is well characterised; it is optimally active at pH 10.5 when assayed on *p*-nitrophenylphosphate but at ~6.8 on caseinate, its optimum temperature is ~37°C. The enzyme is a homodimer of two identical subunits, each of molecular weight ~85 kDa; it contains four atoms of Zn which are essential for activity and is also activated by Mg^{2+} (Linden et al., 1974; Linden and Alais 1976, 1978). AlP is inhibited by metal chelators; the apoenzyme may be reactivated by the addition of one of a number of metals, which is used as the principle of methods to determine very low concentrations of zinc in biological systems. It is also inhibited by inorganic phosphate. The amino acid composition of milk AlP was reported by Linden et al. (1974). It appears that the amino acid sequence of milk AlP has not been reported, but the sequences of human placental and germ cell AlPs show 98% homology (see Hoylaerts and Millan 1991). The sequence of *E. coli* AlP has also been determined and shows 35% homology with human placental AlP and the sequence around the active site is fully conserved. Although milk AlP does not belong to either the placental or intestinal groups of AlP, it is likely that its sequence is generally similar. Models of the tertiary and quaternary structures of *E. coli* AlP were developed by Kim and Wyckoff (1990) and Hoylaerts and Millan (1991). It is likely that the structure of milk AlP is generally similar to that of *E. coli* AlP.

The indigenous AlP in milk is similar to the enzyme in mammary tissue (O'Keefe and Kinsella 1979). The AlP in human milk is similar, but not identical, to human liver AlP (i.e. tissue non-specific type); the difference between the two AlPs is due to variations in the sialic acid content (Hamilton et al., 1979). Unfortunately, a similar comparative study between mammary and liver AlPs has not been reported. Most of the AlP in the mammary gland is in the myoepithelial cells, which may suggest a role in milk secretion; there is much lower AlP activity in the epithelial secretory cells and in milk (Leung et al., 1989; Bingham et al., 1992). The results of the work by Bingham et al. (1992) suggest that there are two AlPs in milk, one of which is from sloughed-off myoepithelial cells, the other originating from lipid microdroplets and acquired intracellularly. The latter is probably the AlP found in the MFGM but unlike XOR it is not a structural component of the MFGM (Leung et al., 1989). Most or all studies on milk AlP have been on AlP isolated from cream/MFGM, i.e. the minor form of AlP in milk; a comparative study of AlP isolated from skimmed milk with that isolated from the MFGM is warranted.

12.11.3 Assay Methods

Kay and Graham (1933, 1935) developed a method based on the inactivation of AlP as an indicator for the adequate pasteurisation of milk. The principle of this method is still used throughout the world and several modifications have been published. The usual substrates are phenylphosphate, *p*-nitrophenyl phosphate or phenolphthalein phosphate, which are hydrolysed to inorganic phosphate and phenol, *p*-nitrophenol or phenolphthalein, respectively:



where XOH = phenol, *p*-nitrophenol or phenolphthalein. The liberated phosphate could be measured but the increase is small against a high background of phosphate in milk. Therefore, in all practical methods, the liberated alcohol is quantified. Reflecting the widespread assay of AlP in routine dairy laboratories, coupled with the need for speed and accuracy, there are more analytical methods for AlP than for any other indigenous milk enzyme. The principal methods are:

- Scharer (1938) used phenyl phosphate as substrate and quantified the liberated (colourless) phenol after reaction with 2,6-dibromoquinonechloroimide, with which it forms a blue complex. The method of Scharer, modified by Sanders and Sager (1946) for application to cheese as well as to milk, uses 2,6-dichloroquinonechloroimide for colour development; this is still the reference method in the USA.
- Kosikowski (1964) modified the method of Sharer by using dialysis rather than a protein precipitant to clarify the phenol-containing solution.
- Aschaffenburg and Mullen (1949) used *p*-nitrophenylphosphate as substrate; the liberated *p*-nitrophenol is yellow at the pH of assay (~10.0). This method, which was modified by Tramer and Wight (1950) by the incorporation of reference coloured standards, is used throughout Europe and in many other countries.
- Huggins and Talalay (1948) and Kleyn (1978) used phenolphthalein phosphate as substrate; the liberated phenolphthalein is red at the alkaline pH of the assay (~10) and hence is easily quantified.
- O'Brien (1966) reacted the phenol liberated from phenylphosphate with 4-aminoantipyrine to form a colourless product which forms a red complex on reaction with potassium ferricyanide; the absorbance of the solution at 505 nm may be determined in an autoanalyser.
- Reynolds and Telford (1967) also developed an automated method based on the dialysis principle of Kosikowski (1964) but using *p*-nitrophenylphosphate as the substrate.
- A fluorogenic aromatic orthophosphoric monoester, Fluorophos (Advanced Instruments, Inc., Needham Heights, MA, USA), has been developed for the determination of AlP in milk and milk products. Hydrolysis of this ester yields a fluorescent compound, 'Fluoroyellow',

the concentration of which is determined fluorometrically (excitation, 439 nm; emission, 560 nm). Fluorometric methods are 100–1,000 times more sensitive than colorimetric assays. A dedicated fluorometer has been developed for the analysis (Advanced Instruments, Inc.). Studies on the fluorometric assay for AlP include Rocco (1990), Eckner (1992), Yoshitomi (2004) and Rampling et al. (2004).

- A chemiluminescent assay for ALP, using adamantyl-1,2-dioxetane phenylphosphate as substrate, was developed for measuring AlP activity in milk by Girotti et al. (1994). A chemiluminescent assay (Paslite) from Charm Sciences Inc., Lawrence, MA, USA, was recently approved by the International Association for Standardisation/International Dairy Federation (ISO 22160/IDF 209) and is (along with the fluorometric test method) documented in the 2009 Pasteurised Milk Ordinance (DHHS-FDA 2007) as acceptable for AlP testing of grade A milk products.
- A rapid, highly sensitive electrochemical method for the determination of AlP using a coupled tyrosinase biosensor was published by Serra et al. (2005). The phenol liberated by AlP is oxidised to quinone by tyrosinase immobilised in a graphite-Teflon-composite electrode containing a Ag/AgCl/KCl reference electrode. The quinone is reduced to catechol at the electrode surface, giving rise to a current that is measured amperometrically. The catechol is reoxidised by tyrosinase to quinone, setting up a redox cycle and giving sensitive detection of AlP. Total analysis time is 5 min, without the need for pre-incubation; the detection limit is 6.7×10^{-14} M AlP.
- The standard colorimetric, fluorometric and chemiluminescent methods (outlined above) for measurement of AlP activity in milk are non-specific, i.e. they are unable to differentiate between bovine AlP and microbial AlP (Painter and Bradley 1997). Enzyme-linked immunosorbent assays (ELISA) have been developed with the objective of differentiating between microbial and milk AlP (Vega-Warner et al., 1999; Chen et al., 2006; Geneix et al., 2007). This type of analytical approach, if used in conjunction with other established approaches (e.g., Fluorophos method), may be useful in determining the level of AlP from microbial sources in milk and dairy products. To the authors' knowledge, there are no approved immunochemical assays available for routine assessment of AlP activity in milk or dairy products; however, such assays would be expected to offer significant potential in the routine, rapid and accurate measurement of AlP activity and in the differentiation of residual native, thermally denatured and reactivated AlP.

A comprehensive review of the various analytical approaches available for measurement of AlP in milk and dairy products, with a focus on validation of milk product pasteurisation was compiled by Rankin et al. (2010).

Most of the studies conducted to date on the quantification of AlP in milk have focused on bovine milk—presumably mainly for commercial reasons, However, the limited research conducted on interspecies comparisons has shown considerable variations in AlP content and activity between species, breeds and individual animals (Raynal-Ljutovaca et al., 2007). Caprine milk has lower AlP activity than bovine milk (Mathur 1974; Williams 1986), while ovine milk is reported to have AlP activity two to three times higher than that of bovine milk, with levels increasing throughout lactation (Scintu et al., 2000). Studies have also shown that AlP in caprine and ovine milks is more susceptible to denaturation on thermal processing than that in bovine milk (Anifantakis and Rosakis 1983; Vamvakaki et al., 2006).

12.11.4 Reactivation of Alkaline Phosphatase

Much work has been focussed on a phenomenon known as 'phosphatase reactivation', first recognised by Wright and Tramer (1953a, b, 1954, 1956), who observed that UHT-treated milk was phosphatase-negative immediately after processing but became positive on storage; microbial phosphatase was shown not to be responsible.

HTST-pasteurised bulk milk does not show reactivation, although some samples from individual cows may. HTST pasteurisation after UHT treatment usually prevents reactivation, which is never observed in in-container sterilised milk. Reactivation can occur following heating at a temperature as low as 84°C for milk or 74°C for cream. The optimum storage temperature for reactivation is 30°C, at which reactivation is detectable after 6 h and may continue for up to 7 days. The greater reactivation in cream than in milk may be due to protection of the enzyme by fat but this has not been substantiated.

A number of attempts have been made to explain the mechanism of reactivation of AlP (see Lyster and Aschaffenburg 1962; Kresheck and Harper 1967; Copius Peereboom 1970; Murthy et al., 1976; Linden 1979; Fox and Morrissey 1981; Andrews et al., 1992; Fox 2003; Fox et al., 2003; Shakeel-Ur-Rehman et al., 2003). There is evidence that the form of the enzyme which becomes reactivated is membrane-bound and several factors which influence reactivation have been established. Mg^{2+} and Zn^{2+} strongly promote reactivation but Sn^{2+}, Cu^{2+}, Co^{2+} and EDTA are inhibitory, while Fe^{2+} has no effect. Sulphydryl (SH) groups appear to be essential for reactivation; perhaps this is why phosphatase becomes reactivated in UHT milk but not in HTST milk. The role of SH groups, supplied by denatured whey proteins, is considered to be chelation of heavy metals, which would otherwise bind to SH groups of the enzyme (also activated on denaturation), thus preventing renaturation. It has been shown that in UHT milk the reactivation rate of AlP is inversely related to oxygen content (Gallusser and Bergner 1981); it was proposed that at high oxygen concentrations, the free SH groups created during the heat treatment are oxidised during storage, preventing SH cross-linking reactions which are critical for the activity of AlP. It has also been proposed that Mg^{2+} or Zn^{2+} cause a conformational change in the denatured enzyme, which is necessary for renaturation. Maximum reactivation occurs in products heated at ~104°C, adjusted to pH 6.5, containing 64 mM Mg^{2+} and incubated at 30°C; homogenisation of products before heat treatment reduces the extent of reactivation (Murthy et al., 1976).

Reactivation of alkaline phosphatase is of considerable practical significance since regulations for HTST pasteurisation specify the absence of phosphatase activity. Methods for distinguishing between renatured and residual native alkaline phosphatase are based on the increase in phosphatase activity resulting from addition of Mg^{2+} to the reaction mixture; various versions of the test have been proposed (see Fox 2003). The official AOAC method is based on that of Murthy and Peeler (1979); however, difficulties are experienced in the interpretation of this test when applied to cream or butter (Kwee 1983; Karmas and Kleyn 1990). Reactivation of AlP is also of significance in the manufacture and analysis of nutritional beverages (e.g., infant formula) fortified with 5′-mononucleotides. In such nutritional beverages, particularly those processed by UHT, reactivated AlP has the potential to convert added nucleotides to nucleosides by dephosphorylation (Gill and Indyk 2007). For this reason, there has been a move in recent years towards development and implementation of HPLC techniques for simultaneous quantification of nucleotides and nucleosides in nucleotide-fortified infant nutritional products.

12.11.5 Significance

Alkaline phosphatase in milk is significant mainly because it is used universally as an index of HTST pasteurisation. However, the enzyme may not be the most appropriate for this purpose (McKellar et al., 1994) because:

- Reactivation of alkaline phosphatase under certain conditions complicates interpretation of the test.
- The enzyme appears to be fully inactivated by temperature *x* time combinations (e.g., 70°C × 16 s), less severe than full HTST conditions (72°C × 15 s).
- The relationship between $\log_{10}$ % initial activity and pasteurisation equivalent deviates slightly from linearity in contrast to the relationship for LPO or GGT (McKellar et al., 1996).

Study of the inactivation kinetics of AlP in milk, as part of the development, evaluation and validation of novel (some being nonthermal) processing technologies, has received attention in recent years, due to its potential use as a marker of the severity of the treatment. These processing technologies have/are being evaluated for the production of extended shelf-life milk or as alternatives to traditional pasteurisation in the production of premium drinking milk products with superior taste and flavour characteristics. Examples of such novel/nonthermal processing technologies include, but are not restricted to, high hydrostatic pressure (HHP), high-pressure homogenisation, pulsed electric fields (PEF), sonication and high-intensity light pulses. High-pressure homogenisation of raw whole bovine milk at 150, 200 or 250 MPa results in 71%, 98% and 100%, respectively, inactivation of AlP of raw milk (Hayes et al., 2005). AlP is quite resistant to HHP, with no inactivation in raw milk after treatment at 400 MPa for 60 min at 20°C (Lopez-Fandino et al., 1996; Huppertz et al., 2005; Rademacher and Hinrichs 2006); HHP at higher temperatures generally increases inactivation of AlP (Seyderhelm et al., 1996; Ludikhuyze et al., 2000). Shamsi et al. (2008) showed that PEF treatment (28–37 kV cm^{-1}) of raw, skimmed milk at 15°C resulted in 24–42% inactivation of AlP. Given the resistance of AlP to inactivation by many of these novel processing technologies, it is likely that new indices of the severity of the treatments will need to be developed for the rapid validation of their effectiveness in the inactivation of microorganisms and/or enzymes.

AlP has the ability to dephosphorylate casein under suitable conditions (Lorient and Linden 1976), but as far as is known, it has no direct technological significance in milk. Perhaps its pH optimum is too far removed from that of milk, especially in acid milk products, although the pH optimum on casein is reported to be ~7 (Lorient and Linden 1976). Moreover, the activity of AlP on casein is inhibited by inorganic phosphate (Lorient and Linden 1976) and whey proteins, particularly β-lactoglobulin (Jasinska et al., 1985). Research conducted over the past 30 years or so focusing on the role of dephosphorylation on the technological properties of casein and casein-based ingredients (e.g., emulsifying and foaming properties) has been done mainly with potato acid phosphatase or calf intestinal alkaline phosphatase (Bingham et al., 1976; Li-Chan and Nakai 1989; Darewicz et al., 2000; Tezcucano Molina et al., 2007; Hiller and Lorenzen 2009).

Proteolysis is a major contributor to the development of the flavour and texture of cheese during ripening. Most of the small water-soluble peptides in cheese are derived from the N-terminal region of α_{s1}- or β-casein; many are phosphorylated and show evidence of phosphatase activity (i.e. they are partially dephosphorylated; see Fox 2003). In cheese made from pasteurised milk, both indigenous acid phosphatase and bacterial phosphatase are probably responsible for dephosphorylation (which is the more important is not clear), but in cheese made from raw milk, e.g., Parmigiano Reggiano or Grana Padano, milk alkaline phosphatase appears to be particularly important (Pellegrino et al., 1997). Further work on the significance of indigenous alkaline and acid phosphatases in the dephosphorylation of phosphopeptides in cheese is warranted.

A recent study by Shakeel-Ur-Rehman et al. (2006) showed that the addition of alkaline phosphatase (of bovine intestinal origin) to pasteurised cheese milk had no quantitative effect on the levels of primary or secondary proteolysis (as measured by water-soluble nitrogen or total free amino acids) in Cheddar-type cheese made therefrom. However, there were qualitative differences in the RP-HPLC peptide profiles of the water-soluble fractions, indicating that AlP activity caused the release of different peptides in the cheese during ripening. This is significant as dephosphorylation of caseins and phosphopeptides can lead to increased peptidase activity during ripening (Ferranti et al., 1997).

12.12 Acid Phosphatase (EC 3.1.3.2)

The occurrence of an acid phosphomonoesterase (AcP) in milk was first reported by Huggins and Talalay (1948) and confirmed by Mullen (1950), who reported that AcP was optimally active at pH

4.0 and was very heat-stable (heating at 88°C for 10 min is required for complete inactivation). The enzyme is not activated by Mg^{2+} (as is AlP), but it is activated slightly by Mn^{2+} and is very strongly inhibited by fluoride. The level of AcP activity in milk is only ~2% that of AlP; activity reaches a maximum 5–6 days *post partum*, then decreases and remains at a low level to the end of lactation (see Andrews et al., 1992).

12.12.1 Isolation and Characterisation

About 80% of the AcP in milk is found in the skimmed milk but the specific activity is higher in cream. Acid phosphatase in milk has been purified to homogeneity by various forms of chromatography, including affinity chromatography (Bingham et al., 1961; Bingham and Zittle 1963; Andews and Pallavicini 1973; Andrews 1976); purification factors of 10,000–1,000,000 have been reported. Adsorption onto Amberlite IRC50 resin is a very effective first step in purification. According to Andrews (1976), all the acid phosphatase activity in skim milk is adsorbed by Amberlite IRC50. However, Flynn (1999) found that only ~50% of the total acid phosphatase in skim milk was adsorbed by Amberlite IRC50, even after re-extracting the skim milk with fresh batches of Amberlite, suggesting that skim milk may contain at least two AcP isozymes. About 40% of the AcP in skim milk partitioned into the whey on rennet coagulation and this enzyme did not adsorb on Amberlite IRC50. The enzyme was partly purified from whey by Flynn (1999).

Flynn (1999) attempted to purify AcP from the MFGM by gel permeation chromatography; sonication and nonionic detergents failed to dissociate the enzyme from the membrane (in agreement with Kitchen 1985). The MFGM enzyme, which does not adsorb on Amberlite IRC50, was much less heat-stable than the acid phosphatase isolated from whey or from skim milk by adsorption on Amberlite IRC50. Overall, it appears that milk contains more than one acid phosphatase.

The AcP activity in milk increases four to tenfold during mastitis. Three isoenzymes are then present, two of which are of leucocyte origin (Andrews and Alichanidis 1975). Using a zymogram technique, Andrews and Alichanidis (1975) reported that milk from healthy cows contains one AcP isozyme while that from mastitic cows contains two additional isozymes which are of leucocyte origin. This may explain the heterogeneity observed by Flynn (1999). The leucocyte isozymes are more thermolabile than the MFGM enzyme and are inactivated by HTST pasteurisation.

Fleming (2000) resolved, by ion-exchange chromatography on DEAE cellulose, the AcP in skimmed milk that adsorbed on Amberlite IRC50 into two fractions, I and II in the proportions of 95:5. These isozymes were generally similar and distinctly different from that isolated from the MFGM by Flynn (1999).

The AcP isolated from skim milk by adsorption on Amberlite IRC50 has been well characterised. It is a glycoprotein with a molecular weight of ~42 kDa and a pI of 7.9. It is inhibited by many heavy metals, F^-, oxidising agents, orthophosphates and polyphosphates and activated by thiol-reducing agents and ascorbic acid; it is not affected by metal chelators (Andrews 1976). It contains a high level of basic amino acids and no methionine.

Since milk AcP is quite active on phosphoproteins, including caseins, it has been suggested that it is a phosphoprotein phosphatase. Although casein is a substrate for milk AcP, the major caseins, in the order α_s $(\alpha_{s1}+\alpha_{s2})>\beta>\kappa$, also act as competitive inhibitors of the enzyme when assayed on *p*-nitrophenylphosphate (Andrews 1974), probably due to binding of the enzyme to the casein phosphate groups (the effectiveness of the caseins as inhibitors is related to their phosphate content).

12.12.2 Assay Methods

Acid phosphatase may be assayed at pH *ca*. 5, on the same substrates as used for AlP. If *p*-nitrophenol phosphate or phenolphthalein phosphate is used, the pH must be adjusted to >8 after incubation to induce the colour of the product, i.e. *p*-nitrophenol or phenolphthalein.

12.12.3 Significance

Although AcP is present in milk at a much lower level than AlP, its greater heat stability and lower pH optimum may make it technologically significant. Andrews (1974) showed that while AcP retains significant activity after HTST pasteurisation at pH 6.7, it does not withstand in-container or UHT sterilisation; however, thermal stability was shown to increase with decreasing pH. Dephosphorylation of casein reduces its heat stability and its ability to bind Ca^{2+}, to react with κ-casein and to form micelles (Bingham et al., 1976; Tezcucano Molina et al., 2007).

As discussed under AlP, several small partially dephosphorylated peptides have been isolated from Cheddar, Parmigiano Reggiano and Grana Padano cheese (de Noni et al., 1997; Ferranti et al., 1997; Pellegrino et al., 1997; Singh et al., 1997). However, it is not known whether indigenous or bacterial acid phosphatase is mainly responsible for dephosphorylation in cheese made from pasteurised milk. It is claimed (see Fox 2003; Shakeel-Ur-Rehman et al., 2003; Akuzawa and Fox 2004) that alkaline phosphatase is mainly responsible for dephosphorylation of peptides in raw milk cheese. Dephosphorylation may be rate-limiting for proteolysis in ripening cheese since most proteinases and peptidases are inactive on phosphoproteins or phosphopeptides (Schormuller et al., 1960).

Given that AcP activity in milk increases four to tenfold during mastitic infection, the enzyme could be used as an index of mastitis; however, other enzymes (e.g., *N*-acetylglucosaminidase) are more effective markers.

The suitability of AcP as an indicator enzyme for super-pasteurisation of milk has been assessed (Griffiths 1986; Andrews et al., 1987); it is not as useful for this purpose as some alternatives, e.g., γ-glutamyl transpeptidase or LPO.

AcP is much less resistant to HHP than AlP, with the majority of AcP activity being lost within 10 min during treatment at 500 MPa, whereas AlP requires pressures in excess of 800 MPa to be inactivated at room temperature. This difference has been suggested by Balci et al. (2002) as useful in discriminating between heat- and pressure-treated milks.

12.13 Nucleases

12.13.1 Ribonuclease (EC 3.1.4.22)

Ribonucleases (RNase) catalyse cleavage of the phosphodiester bond between the 5′-ribose of a nucleotide and the phosphate group attached to the 3′ position of ribose of an adjacent pyrimidine nucleotide, forming a 2′, 3′ cyclic phosphate, which is then hydrolysed to the corresponding 3′-nucleotide phosphate. RNases of various origin and with different biological functions have been purified and characterised. They form a superfamily, which has been the subject of several reviews, including those by Barnard (1969), Adams et al. (1986), D'Alessio and Riordan (1997) and Bientema and Zhao (2003) and in a series of articles in the journal, *Cellular and Molecular Life Sciences* (Anon 1998). RNase occurs in various tissues and secretions, including milk (see Barnard 1969). Bovine pancreatic RNase A has been studied in great detail; it was the first enzyme to have its complete amino acid sequence determined (Smyth et al., 1963), and early studies on its tertiary structure were reported by Kartha et al. (1967). It contains 124 amino acid residues, with a calculated molecular weight of 13,683 Da, and has a pH optimum of 7.0–7.5.

Although Zittle and DellaMonica (1952) reported that fractions of bovine milk showed phosphodiesterase activity when RNA was used as substrate, the first study on the indigenous RNase in milk appears to be that of Bingham and Zittle (1962). These authors reported that bovine milk contains a much higher level of RNase than the blood serum or urine of human, rat or guinea pig and that most or all of the activity is in the serum phase; bovine milk could potentially serve as a commercial source of RNase. Like pancreatic RNase, the RNase in milk is optimally active at pH 7.5 and is more heat-stable at acid pH values than at pH 7; in acid whey, adjusted to pH 7, 50% of RNase activity was lost on heating at 90°C for 5 min and 100% after 20 min, but it was completely stable in whey at pH 3.5 when heated at 90°C for 20 min (Bingham and Zittle 1962). The enzyme was purified 300-fold by adsorption

on Amberlite IRC-50 resin with desorption by 1 M NaCl, followed by precipitation with cold (4°C) acetone (46–66% fraction). The partially purified enzyme showed no phosphodiesterase activity on Ca [*bis* (*p*-nitrophenyl phosphate)]$_2$ as substrate.

The RNase in bovine milk was further purified from acid whey by Bingham and Zittle (1964), using the same general procedure but with elution from Amberlite IRC-50 using a NaCl gradient, which resolved two isoenzymes, A and B, at a ratio of about 4:1, as for pancreatic RNase. Amino acid analysis, electrophoresis and immunological studies showed that milk RNase is identical to pancreatic RNase (Bingham and Zittle 1964). It is presumed that the RNase in milk originates in the pancreas and is absorbed through the intestinal wall into the blood, from which it enters milk. Intestinal absorption of pancreatic RNase (13,683 Da) was demonstrated in rats by Alpers and Isselbacher (1967), showing that it is possible for proteins of this size to be absorbed into the blood stream, although the level of RNase activity in milk is considerably higher than in blood serum, which suggests active transport (Bingham and Zittle 1962).

Ribonucleases A and B were isolated from bovine milk by Bingham and Kalan (1967) essentially by a scaled-up version of the procedure of Bingham and Zittle (1964) and including a gel permeation step. Two other isoenzymes, C and D, were demonstrated but not purified. Milk RNase A was shown by various criteria to be identical to pancreatic RNase A, but milk RNase B was shown to differ from both milk and pancreatic RNase A and pancreatic RNase B. All four isozymes had the same amino acid composition, but the two RNase B isozymes are glycoproteins, which differed in sugar content and chromatographic behaviour; both RNase A isozymes were free from carbohydrate.

Chandan et al. (1968) reported that bovine milk contains about three times as much RNase as human, ovine or caprine milk and that porcine milk contains a very low level of RNase. The same group (Dalaly et al., 1970) purified RNase from human milk; the principal isozyme contained no carbohydrate but the minor one was a glycoprotein. The enzyme hydrolysed RNA, polycytidylic and polyuridylic acids, but not polyadenylic or polyguanylic acids or DNA. Dalaly et al. (1970) considered milk RNase to be generally similar to bovine pancreatic RNase. Further characterisation of the two human milk isozymes was reported by Dalaly et al. (1980).

Gupta and Mathur (1989a) reported a single peak of RNase activity for goats' milk following chromatography on Amberlite IRC-50 resin; both bovine and buffalo milks showed two peaks of activity after the same procedure. The molecular weight of goat milk RNase was reported to be 29,500 Da and the enzyme showed maximum activity at 50°C and pH 9; the large differences between these values and the corresponding characteristics of bovine RNase have not been explained. According to Gupta and Mathur (1989b), goats' milk contains about one third as much RNase as bovine or buffalo milk. The literature on nucleases, including RNase, in milk has been reviewed by Stepaniak et al. (2003).

The possible immunological and nutritional effects of RNase in milk were investigated by Meyer et al. (1987a). Three isoenzymes were isolated from bovine milk by cation exchange chromatography on phosphocellulose: RNase A and B, previously reported by Bingham and Zittle (1964), and an isoenzyme termed RNase II-1, in the ratio 70:30:1. RNase II-1 differed from A and B in being more heat-stable and also in its inability to hydrolyse polycytidylate. [The classification nomenclature used by Meyer et al. (1987a) for RNases was based on immunological reaction and conflicts with that of the International Union of Biochemistry, which designates pancreatic ribonuclease (and milk RNase) as Ribonuclease I (EC 3.1.27.5)]. Meyer et al. (1987a) reported that bovine colostrum has three times as much total RNase activity as mature milk and 10–15 times more RNase II-1. RNase activity is also elevated in mastitic milk, to more than twice the normal level. Considering that tissue RNases also increase during infection, Meyer et al. (1987b) suggested that the RNase in milk may play a role in protecting the neonate against microbial infection.

Little or no RNase activity survives UHT heat treatment (121°C for 10 s) but about 60% survives

heating at 72°C for 2 min (Meyer et al., 1987a) or at 80°C for 15 s (Griffiths 1986). RNase activity in raw or heat-treated milk is stable to repeated freezing and thawing and to frozen storage for at least a year (Meyer et al., 1987a).

A high molecular weight (80 kDa) RNase (hmRNase) was purified from human milk by Ramaswamy et al. (1993) and characterised as a single-chain glycoprotein, with a pH optimum in the range 7.5–8.0. It was more heat-labile than bovine RNase A and was considered to be an isoform of lactoferrin, due to similarities in physical, chemical and antigenic properties; however, RNase has no iron-binding capacity and lactoferrin has no RNase activity. It was speculated that hmRNase is synthesised in the mammary gland and passes into milk, rather than being transferred from blood, as are RNase A and B (Bingham and Zittle 1964).

Ramaswamy et al. (1993) reported that the incidence of breast cancer is about three times higher in Parsi women in Western India than in other Indian communities and that the level of RNase in their milk is lower than normal. It was suggested that RNase may serve as a marker for the risk of breast cancer.

Research has intensified in recent years on the antiviral and antitumour activities of RNases. With the knowledge that the antitumour activity of bull semen RNase depends on its dimeric structure, Piccoli et al. (1999) engineered human pancreatic RNase from a monomeric to a dimeric form. The engineered protein was enzymatically active and selectively cytotoxic for several malignant mouse and human cell lines. This could offer a less toxic alternative to chemotherapeutic agents in the treatment of cancer patients. An amphibian RNase, called onconase, has shown success in clinical trials on cancer patients (Mikulski et al., 1993; Saxena et al., 2002). Recent research using recombinant DNA technology has shown that mouse and human RNase have bactericidal activity, suggesting a role for RNase activity in host defense in the intestinal epithelium (Hooper et al., 2003).

Lee-Huang et al. (1999) identified RNase in the urine of pregnant women as a factor responsible for activity against type 1 HIV virus. Pancreatic RNase was also effective in blocking HIV replication, creating an exciting new avenue for research on the treatment of AIDS. McCormick et al. (1974) found that RNase protects milk from viruses by inhibiting the action of RNA-dependent DNA polymerase and thus preventing viral replication. Perhaps RNase can inhibit bacteriophage, which inhibits the growth of starter cultures in cheesemaking; such a study seems warranted. Based on the similarity of its structure to angiogenin, a protein which induces blood vessel formation in tumours, Roman et al. (1990) suggested that growth promotion may be a biological function of RNase in milk and colostrum. Although RNase has no technological significance in milk, which contains very little RNA, it may have significant biological functions.

12.13.2 Catalytic Antibodies (Abzymes) with Oligonuclease Activity

The term abzyme is used to describe antibodies with enzymatic activity, including nuclease activity. The first catalytic antibodies were produced in 1986 (Lerner et al., 1991), with the term abzyme, derived from 'antibody enzyme' being used routinely to describe such antibodies. Catalytic antibodies are relatively slow catalysts, with turnover numbers 10^3–10^6 times lower than is common for enzymes (Kirby 1996). Catalytic antibodies capable of catalysing a broad spectrum of chemical reactions have been produced (Lerner et al., 1991; Janda 1994; Shchurov 1997). The selectivity of antibodies is usually higher than that of enzymes, which has important implications for the biological function of abzymes. The abzymes produced to date are generally produced by immunisation with a transition state analogue coupled as a hapten to a carrier protein (Kirby 1996; Fletcher et al., 1998). The presence of DNA- and RNA-hydrolysing antibodies has been demonstrated in the milk of healthy women (Kanyshkova et al., 1997; Buneva et al., 1998). Human milk also contains secretory immunoglobulin A (sIgA) which can catalyse the hydrolysis of RNA and DNA (Kit et al., 1995; Nevinsky et al., 2000). It is likely that such abzymes are present in human milk to confer a protective role

(e.g., antibacterial or antiviral activity) for infants, given that the immune system of infants is not fully developed in the early stages of life. The literature on catalytic antibodies has been reviewed by Lerner et al. (1991), Benkovic (1992), Suzuki (1994), Kirby (1996), Shchurov (1997) and Stepaniak et al. (2003).

12.13.3 5′-Nucleotidase (EC 3.1.3.5)

5′-Nucleotidase catalyses the hydrolysis of 5′-nucleotides; the enzyme is a component of the MFGM (Patton and Trams 1971) and has been purified from acid whey (Caulini et al., 1972) and from the MFGM (Huang and Keenan 1972). The enzyme may be purified from the MFGM using detergent treatment, $(NH_4)_2SO_4$ fractionation, heat treatment, sonication and chromatography on Sepharose-4B. Such an approach yields two fractions (designated V and VI) with 5′-nucleotidase activity (Huang and Keenan 1972). The two isoenzymes differ in phospholipid content, substrate specificity and kinetic properties. 5′-Nucleotidease has optimum activity at pH 7.0–7.5 (Caulini et al., 1972; Huang and Keenan 1972) and 69°C (Huang and Keenan 1972) and it does not require a metal cofactor. 5′-Nucleotidase activity is suitable for use as a marker in studying secretory mechanisms for the milk fat globules and biogenesis of MFGM material. Heating at 60°C for 30 min or 75°C or 80°C for 15 s reduces the activity of 5′-nucleotidase by 20%, 40% and 97%, respectively (Huang and Keenan 1972; Andrews et al., 1987). While milk contains 5′-mononucleotides (i.e. substrates for 5′-nucleotidase activity), such indigenous nucleotides appear to be resistant to dephosphorylation (Gill and Indyk 2007), albeit by mechanisms which are still poorly understood.

12.14 Lysozyme (EC 3.1.2.17)

According to Whitney (1958), Shahani et al. (1962) and Chandan et al. (1965), the presence of natural antibacterial factor(s) in fresh raw bovine milk was reported by Kitasoto in 1889 and by Fokker in 1890. These inhibitors are now called lactenins, one of which is LPO.

Fleming (1922, 1929) identified an antibacterial agent in nasal mucus, tears, sputum, saliva and other body fluids which caused lysis of many types of bacteria (*Micrococcus lysodeikticus* was used for assays). He showed that it was an enzyme, which he called lysozyme. [According to Jolles and Jolles (1967), it had been known since 1893 that tears possessed bactericidal activity.] Fleming (1922, 1929) found that chicken egg white is a particularly rich source of lysozyme; it constitutes ~3.5% of egg-white protein and is the principal commercial source of lysozyme. Chicken egg-white lysozyme (EWL) is referred to as lysozyme 'c'; a second type of lysozyme, 'g', is present in the egg white of the domestic goose; the two lysozymes differ in molecular weight and amino acid composition. EWL is easily purified and has been studied extensively as a model protein for structure, dynamics and folding; the literature has been reviewed by Kato (2003).

Fleming (1922, 1929) did not include milk among the several fluids in which he found lysozyme but Bordet and Bordet (1924) reported that the milk of several species contains lysozyme and that human milk is a comparatively rich source. The situation regarding bovine milk was less clear; some workers, including Fleming (1932), reported that bovine milk contains lysozyme but others did not find it (see Shahani et al., 1962). Lysozyme has been isolated from the milk of a wider range of species than any other milk enzyme. This may reflect the perceived importance of lysozyme as a protective agent in milk or it may be because it can be isolated from milk relatively easily. While the milk lysozymes are generally similar, there are substantial differences, even between closely related species, e.g., cow and buffalo.

Lysozyme (also called muramidase, mucopeptide *N*-acetyl-muramyl hydrolase) is a widely distributed enzyme which lyses certain bacteria by hydrolysing the β $(1 \rightarrow 4)$-linkage between muramic acid and *N*-acetylglucosamine of mucopolysaccharides in the bacterial cell wall. The presence and activity of lysozyme is normally assayed for by the lysis of a culture of *M. lysodeikticus*, measured by a decrease in

turbidity (e.g., Manas et al., 2006), but it can also be assayed by enzyme-linked immunosorbent assay techniques using monoclonal or polyclonal antibodies (Rauch et al., 1990; Yoshida et al., 1991; Besler 2001; Schneider et al., 2010b), reversed-phase high-performance liquid chromatography with fluorescence detection (Pellegrino and Tirelli 2000), liquid chromatography-mass spectrometry (LC-MS), immunocapture mass spectrometry or surface-enhanced mass spectrometry (Schneider et al., 2010a). In recent years, the increasing incidence of cases describing allergic reactions to lysozyme present in food products has refocused attention on comparison and development of rapid, specific, sensitive and reliable methods for the detection and quantification of lysozyme in food matrices (Kerkaert et al., 2010; Jimenez-Saiz et al., 2011; Schneider et al., 2011).

Lysozyme was isolated from human milk by Jolles and Jolles (1961), who believed that bovine milk was devoid of lysozyme; human milk lysozyme (HML) was found to be generally similar to EWL. Variability in the level of lysozyme in human milk and its heat stability were studied by Chandan et al. (1964) and the isolation procedure was improved by Jolles and Jolles (1967) and Parry et al. (1969); a method for the simultaneous isolation of RNase and lysozyme from human milk was reported by Dalaly et al. (1970).

According to Chandan et al. (1965), lysozyme had by then been found in the milk of many other species, e.g., donkey, horse, dog, sow, cat, rat, rabbit, llama and rhesus monkey, but no lysozyme or only traces were found in the milk of goat, sheep and guinea pig; they did not mention bovine milk although a low and variable level of lysozyme had been found in bovine milk by Shahani et al. (1962). According to Chandan et al. (1968), porcine milk is devoid of lysozyme but this has not been confirmed (see Wagstrom et al., 2000). Equine milk has a very high ability to inhibit bacterial growth, which is probably due to its high level of lysozyme activity.

Bovine milk lysozyme (BML) was isolated and characterised by Chandan et al. (1965), Dalaly et al. (1970) and Eitenmiller et al. (1971, 1974, 1976). Equine milk lysozyme was isolated and characterised by Jauregui-Adell (1971, 1975) and Jauregui-Adell et al. (1972). Human and equine milks are exceptionally rich sources of lysozyme, containing 400 and ~800 mg/L, respectively (3,000 and 6,000 times the level in bovine milk); these levels represent ~4% and ~3% of the total protein in human and equine milk, respectively (Chandan et al., 1968; Jauregui-Adell 1975). Asinine milk contains about the same level of lysozyme as equine milk (Civardi et al. 2002). Although lysozyme is a lysosomal enzyme, it is found in soluble form in many body fluids (tears, mucus, egg white) and the lysozyme in milk is usually isolated from whey, indicating that it is in solution.

In addition to the lysozyme in human, equine and bovine milk, lysozyme has been isolated and partially characterised from the milk of several other species: baboon (Buss 1971), camel (see Benkerroum et al., 2004), buffalo (Priyadarshini and Kansal 2002, 2003) and dog (Watanabe et al., 2004). The reported properties of these lysozymes are generally similar to those of HML, but there are substantial differences, even between the lysozymes of closely related species, e.g., cow and buffalo.

The pH optimum of HML, BML and EWL is 7.9, 6.35 and 6.2, respectively (Chandan et al., 1965; Parry et al., 1969). According to Eitenmiller et al. (1971, 1976) and Friend et al. (1972), BML has a molecular weight of 18 kDa compared with 15 kDa for HML and EWL, and its amino acid composition and immunological properties are considerably different from those of the latter two lysozymes. White et al. (1988) isolated BML and found that it resembled closely the BML studied by Chandan et al. (1965), including a mass of ~18 kDa. However, when they analysed their preparation by RP-HPLC it resolved into two peaks, only the smaller of which had lysozyme activity; the larger peak was inactive and had a high molecular weight. White et al. (1988) suggested that the apparent relatively high molecular weight of BML reported by Eitenmiller et al. (1971, 1974, 1976) was due to a high molecular weight impurity. A more thorough study of a homogeneous preparation of BML appears warranted.

The complete amino acid sequence of HML and EWL were reported by Jolles and Jolles (1972). Although highly homologous, the sequences showed several differences; HML consists of 130 amino acid residues, compared with 129 in EWL, the extra residue in the former being Val_{100}. The amino acid sequence of equine milk lysozyme was reported by McKenzie and Shaw (1985); the molecule consists of 129 amino acid residues, like EWL, with a mass of 14,647 Da. It showed only 51% homology with HML and 50% homology with EWL. The partial sequence of BML reported by White et al. (1988) showed differences between EWL, HML and BML and from lysozymes of other animal tissues (Ito et al., 1993). The three-dimensional structure of EWL was reported by Blake et al. (1965); Johnson (1998) reviewed further studies on the structure of lysozyme.

The amino acid sequence of lysozyme is highly homologous with that of α-lactalbumin (α-la), a whey protein which is an enzyme modifier in the biosynthesis of lactose. The similarities in primary structure, gene sequence and three-dimensional structure of α-la and c-type lysozymes are described by McKenzie and White (1991). α-La binds a Ca^{2+} in an Asp-rich loop but most c-type lysozymes do not bind a Ca^{2+}, equine and canine milk lysozymes being exceptions (Tada et al., 2002; Watanabe et al., 2004).

All lysozymes are relatively stable to heat at acid pH values (3–4) but are relatively labile at pH >7. More than 75% of the lysozyme activity in bovine milk survives heating at 75°C × 15 min or 80°C × 15 s, and therefore it is affected little by HTST pasteurisation. HML and BML are inactivated by mercaptoethanol; the reduced enzyme can be reactivated by diluting the desalted reduced protein in 0.1 M Tris–HCl buffer (pH 8.5). The activity of reoxidised BML and HML were ~330% and 84%, respectively, of the native enzyme (Friend et al., 1972).

The effects of specifically modifying residues in EWL, HML and BML showed that the first two behaved generally similarly but BML appeared to be quite different; e.g., modifying Trp strongly inhibited EWL and HML but BML was inhibited only slightly (Friend et al., 1975). These authors concluded that BML differs from most lysozymes of animal origin but resembled plant lysozymes, especially those from fig or papaya. These differences do not seem to have been investigated further.

The most significant physiological role of lysozyme is to act as a bactericidal agent, evidence of which has been available for decades (e.g., Fleming 1922). The bactericidal effect of lysozyme against Gram-positive microorganisms is partially dependent on its lytic activity on the cell wall (Jimenez-Saiz et al., 2011), while research conducted over the last 10 years or so also implicates a nonenzymatic mechanism of action (Ibrahim et al., 2002; Masschalck and Michiels 2003). Much research has also been conducted on various means of enhancing the antibacterial activity of lysozyme against Gram-negative bacteria; examples of such approaches include HHP (Masschalck et al., 2002), ultrafiltration (Cegielska-Radziejewska et al., 2003), disulphide bond reduction and covalent attachment of various components such as polysaccharides, fatty acids and peptides (Masschalck and Michiels 2003). Research on this topic has suggested that the nonenzymatic mechanism of action of lysozyme may involve disruption of normal electrostatic interactions between divalent cations and components of the outer cell membrane of Gram-negative bacteria (Ibrahim 1998; Ibrahim et al., 1997). In addition to bactericidal activity, biological functions of lysozyme, such as immunomodulatory, antiviral and anti-inflammatory activity, have also been reported (Lesnierowski and Kijowski 2007).

In the case of milk, lysozyme may simply be a 'spill-over' enzyme or it may have a definite protective role. If the latter is true, then the exceptionally high level of lysozyme in human and equine milk may be significant. The specific activity of human lysozyme is approximately ten times greater than that of bovine lysozyme. HML also has approximately three times more lytic activity than that of EWL due to the fact that it possesses a greater positive charge than the latter (Parry et al., 1969). Breast-fed infants generally

experience a lower incidence and severity of infections and gastrointestinal difficulties than formula-fed infants. The role of HML in reducing microbial infections in the gastrointestinal tract of breast-fed infants has been studied extensively (Lonnerdal 1985). Addition of lysozyme to infant formula has been shown to reduce the incidence of gastroenteritis and allergies and to increase the beneficial gastrointestinal microflora (Birch and Parker 1980). Given these significant biofunctionalities, but due to the lack of availability, some recent research has focused on transgenic production of lysozyme (Maga et al., 1994; Yu et al., 2006; Scharfen et al., 2007; Yang et al., 2011).

One might expect that, owing to its bactericidal effect, indigenous milk lysozyme would have a beneficial effect on the shelf-life of milk; there are limited reports to support this expectation, e.g., a study conducted on the shelf-life of goat milk containing recombinant human lysozyme (Maga et al., 2006). Research is also underway on the effects of lysozyme addition as part of an overall nonthermal hurdle approach to microbiological control of milk and dairy products. Early research has demonstrated synergistic effects of combining lysozyme with high-intensity PEF technology for inactivation of *Staphylococcus aureus* in skim milk (Sobrino-Lopez and Martin-Belloso 2008). Other preliminary research has shown that the bactericidal activity of chicken EWL against *Listeria monocytogenes*, in media, may be enhanced by high-pressure homogenisation at 100 MPa (Iucci et al., 2007).

Exogenous lysozyme may be added to milk in the manufacture of several hard and semihard cheese varieties, e.g., Gouda, Edam, Emmental and Parmigiano Reggiano, as an alternative to nitrate to prevent the growth of *Clostridium tyrobutyricum* which can cause late gas blowing and off-flavour defects during ripening. This is probably the most widespread commercial application for chicken EWL, although it is also used to control malolactic fermentation of wine (Proctor and Cunningham 1988; Tirelli and De Noni 2007). As eggs and egg products are major food allergens, EC legislation (2003/89/EC, Annex IIIa amending Directive 2000/13/EC) mandates that the use of lysozyme as an additive needs to be declared on the ingredient/product label.

12.15 Amylase (α-Amylase EC 3.2.1.1, β-Amylase EC 3.2.1.2)

Amylase (diastase) was one of be first indigenous enzymes identified in milk; according to Sato (1920), Bechamp 'isolated' an amylase from human milk in 1883. During the next 40 years, several workers reported that the milk of several species contains an amylase but several other authors reported that they do not. Sato (1920) reported that all samples of raw milk and cheese assayed by him contained amylase and he concluded that the enzyme is produced in the mammary gland. Richardson and Hankinson (1936) also concluded that the amylase in milk is indigenous and that α-amylase is the principal enzyme, with a lesser amount of β-amylase; the enzymes partition mainly into skimmed milk and whey. A highly concentrated preparation of α-amylase was obtained from whey by Guy and Jenness (1958). There appears to have been no further work on the isolation of amylase from bovine milk. Milk amylase is quite labile to heat and inactivation was proposed as a reliable index of the intensity of heat treatment applied to milk (Orla-Jensen 1929; Gould 1932).

Human milk and colostrum contain 25–40 times more α-amylase than bovine milk; however, there is at least a tenfold variation in the amylase activity in individual cow milk samples (Stejskal et al., 1981; deWit et al., 1993). The α-amylase in milk is similar to salivary amylase but different from pancreatic amylase; it appears that during pregnancy, the production of α-amylase is switched from saliva to the mammary gland (Stejskal et al., 1981). The amylase in human milk is a major contributor to the ability of human breast-fed infants to digest starch.

α-Amylase was purified from human milk by gel permeation chromatography and its stability to pH and pepsin determined (Lindberg and Skude 1982). These investigators reported that

the level of α-amylase in human milk is 15–140 times higher in human milk than in blood plasma, suggesting that it is not transferred from the blood but is synthesised in the mammary gland. Since bovine milk contains no starch and only low levels of oligosaccharides, the function of amylase in milk is unclear. Human milk, also, does not contain starch but it contains up to 130 oligosaccharides, at a total concentration up to 150 mg/L (e.g., Newburg and Neubaurer 1995; Miller and McVeigh 1999; Urashima et al., 2001). These oligosaccharides are built up from lactose and contain unusual monosaccharides (e.g., fucose and *N*-acetylneuraminic acid) linked by unusual glycosidic bonds; therefore, it is unlikely that α-amylase, which is highly specific for α (1→4) glycosidic bonds linking glucose molecules, will hydrolyse the oligosaccharides in milk (Gnoth et al., 2002). Since human babies secrete low levels of salivary and pancreatic amylases (0.2–0.5% of the adult level; Hamosh 1988), the high level of amylase activity in human milk may enable them to digest starch in infant formulae (Lindberg and Skude 1982; Heitlinger et al., 1983; Hamosh 1988; deWit et al., 1993). By hydrolysing the polysaccharides in the cell wall of bacteria, it has been suggested that milk amylase may have antibacterial activity (see Lindberg and Skude 1982). The amylase activity of human milk is an active area of research at present but there appears to be little or no recent research on the amylase in bovine milk or that of other species. It seems reasonable to suggest that the isolation, characterisation and significance of amylase from the milk of other species warrant investigation. El-Fakharany et al. (2009) isolated and characterised a β-amylase from camel milk [they appear not to have assayed for α-amylase activity]; it had a molecular mass of 61 kDa, which differed from that of other animal amylases, including camel pancreatic amylase. Moatsou (2010) reported that there are no publications on amylase in ovine or caprine milk and the current authors have found no reports on amylase in the milk of other species.

Human milk also contains abzymes [antibodies with enzymatic activity] with amylotic activity, especially in the IgG and IgA fractions (Kulminskaya et al., 2004).

12.16 β-*N*-Acetylglucosaminidase (E.C. 3.2.1.30)

β-*N*-Acetylglucosaminidase (NAGase) hydrolyses terminal, nonreducing *N*-acetyl-β-D-glucosamine residues from *N*-acetyl-β-D-glucosaminides, including glycoproteins and fragments of chitin. However, NAGase is not specific for *N*-acetyl-β-D-glucosaminides; since it can also hydrolyse *N*-acetyl-β-D-galactosaminides, it has been recommended (Cabezas 1989) that the enzyme should be called *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52).

NAGase is thought to be a lysosomal enzyme (Sellinger et al., 1960) which originates principally from mammary gland epithelial cells and, to a lesser extent, from somatic cells. The first report on NAGase in milk appears to be that of Mellors (1968), who purified (~10-fold increase in specific activity) the enzyme from separator slime. More than 95% of NAGase in milk is in the skimmed milk. The enzyme is optimally active at 50°C and pH 4.2. Mellors (1968) suggested that NAGase should be a convenient index of mammary gland infection. The effectiveness of NAGase as an indicator of mastitis was demonstrated by Kitchen (1976), Kitchen and Midleton (1976) and Kitchen et al. (1978). Since then, there have been numerous studies on the reliability of NAGase as a marker of mastitis (Mattila 1985; Pyörälä and Pyörälä 1997; Bansal et al., 2005; Larsen et al., 2010a, b; Barth et al., 2010). A field test for mastitis based on NAGase activity has been developed using chromogenic *N*-acetyl-β-D-glucosamine-*p*-nitrophenol as substrate; hydrolysis yields *p*-nitrophenol, which is yellow at alkaline pH (Kitchen and Midleton 1976). NAGase activity is also high in colostrum.

NAGase is inactivated by HTST pasteurisation (70–71°C × 15–18 s) and Andrews et al. (1987) proposed that NAGase would be a suitable indicator enzyme for assessing heat treatment in the range 65–75°C × 15 s. With the objective of developing a test to determine the heat load to which cheese milk had been subjected, Ardo et al. (1999) compared the thermal inactivation of alkaline phosphatase (AlP), NAGase and GGT. As AlP was considered to be

too heat-sensitive and GGT too heat-stable to meet the objective, NAGase was considered to be the most suitable.

Although NAGase is a lysosomal enzyme, it occurs mainly in the whey fraction (82% of total activity; Kitchen et al., 1978), from which it has been isolated by various forms of chromatography. Two isozymes of NAGase, A and B, differing in molecular weight, i.e. 118 and 234 kDa, respectively, and charge were isolated from bovine mammary tissue by Kitchen and Masters (1985). Each isoenzyme dissociates into two dissimilar subunits of mass 55 and 25 kDa, on treatment with 2-mercaptoethanol and sodium dodecyl sulphate.

12.17 Aldolase (EC 4.1.3.13)

Aldolase reversibly hydrolyses fructose 1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate; it is a key enzyme in the glycolytic pathway. The presence of aldolase in milk was first reported by Polis and Shmukler (1950), who partially purified it. Although most (66%) of the aldolase in milk is in the skimmed milk (Kitchen et al., 1970), it is also found in the cream/MFGM (Polis and Shmukler 1950; Erwin and Randolph 1975; Keenan et al., 1988; Keenan and Dylewski 1995; Keenan and Mather 2006). According to Blanc (1982), the aldolase is located in the cytoplasm of the mammary cells, from which the enzyme in milk presumably originates, although some may be from blood.

It has been suggested (Dwivedi 1973) that aldolase plays a role in flavour development in dairy products. There appear to have been no recent publications on milk aldolase. The aldolase from rabbit muscle is a homotetramer of 161 kDa (4×40 kDa) with a pH optimum of ~7.0. The literature on aldolase was reviewed by Horecker et al. (1972).

12.18 Conclusions

As a biological fluid, it is not surprising that milk contains enzymes—approximately 70 have been identified to date. Many of these enzymes are present at quite high concentrations, and those that are easily assayed were studied long before the proteinaceous nature of enzymes was recognised, even before the term 'enzyme' was coined. Being a fluid, it was relatively easy to purify and study the indigenous enzymes of milk. Some of the indigenous enzymes in milk are significant for the protection and/or nutrition of the neonate but most are not important and none is essential. However, many are very significant in dairy technology as a cause of spoilage or as indicators of quality or history. The lipids and proteins of milk are susceptible to the action of milk enzymes, generally with negative effects; however, lactose is not a substrate for any of these enzymes.

Although the indigenous enzymes in milk have been studied since 1881, they are still very active research subjects. The focus of attention has changed many times during the past 130 years, mainly as dairy processing technologies changed and new dairy-based products were developed. Not surprisingly, most research has been on bovine milk, with human milk also receiving considerable attention. Some enzymes in the milk of other commercially important domesticated species have been studied but little or no research has been done on the enzymes of most species. Available data indicate that there are some very large interspecies differences in the levels of many enzymes, some of which are physiologically and/or technologically important. The reasons for these interspecies differences are unknown and may reflect differences in the mechanism of synthesis or secretion of milk constituents.

Considering the great diversity of mammals, it is not an exaggeration to suggest that the study of milk enzymology offers an almost inexhaustible number of research projects.

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13 Interspecies Comparison of Milk Proteins: Quantitative Variability and Molecular Diversity

P. Martin, C. Cebo, and G. Miranda

13.1 Introduction

Milk is a complete and complex food suited to the specific offspring requirements for growth and development. Its composition is the result of a long and slow adaptive evolution process that started 150 million years ago, long before the domestication of ruminants, which took place *ca*. 10,000 years ago in the Fertile Crescent region (Zeder 2008).

There is increasing substantial evidence that milk contains many health-promoting compounds, impacting physiological functions or reducing disease risk. This statement is true for the main milk components, such as lipids, carbohydrates (including oligosaccharides) and proteins as well as for minerals or vitamins. As far as milk proteins are concerned, numerous substantiated or potential bioactive proteins have been found, and many others remain to be identified either as intact protein or as derived peptides, encrypted in the sequence of milk proteins. This is probably one of the greatest challenges facing milk science in the immediate future: to provide the food industry and consumers with the basis for health-promoting properties before their inclusion as ingredients into functional foods.

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Milk proteins are found mostly in the aqueous phase, either in soluble (whey proteins, of which few are synthesised in the mammary epithelial cells (MEC), whilst the large majority have a serum origin) or colloidal (caseins) states, but also in the lipid phase, associated with the milk fat globule membrane (MFGM).

It has been shown that caseins, which can account for more than 80% of milk proteins, have evolved rapidly and are highly divergent proteins across mammalian milks. However, it appears that although both copy number and sequence variation contribute to the diversity of milk protein composition across species, milk and mammary genes are more highly conserved, on average, than other genes in the bovine genome (Lemay et al., 2009). For a long time, we have believed that this feature meant that they were devoid of biological functions and were designed only to ensure amino acids supply and phosphate and calcium absorption. This is no longer true since we now know that peptides displaying proven biological activity are encrypted within caseins as well as whey proteins such as α-lactalbumin which can attain new functions by changing its three-dimensional structure (Pettersson-Kastberg et al., 2009).

Regarding the casein fraction, several tens of genetic variants have been characterised so far in cow, ewe and goat milks (Table 13.1; for more details, see Chap. 15). The past 20 years have seen remarkable progress in the understanding of the structure and function of milk protein genes. Developments in molecular biology, genomics and

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Table 13.1 Genetic variants of milk proteins in the ruminant species: an overall picture of our present knowledge

	CSN1S1 (α_{s1}-casein)	CSN1S2 (α_{s2}-casein)	CSN2 (β-casein)	CSN3 (κ-casein)	β-lactoglobulin	α-lactalbumin
Cattle	7 variants, A to G (+H)	4 variants, A to D	**9 variants, (+4) A^1, A^2, A^3, B to G (B2, A^4, A^3Mong)**	4 variants, (+5) A, B, C/D & E (F to J)	7 variants, A to G	3 variants, A to C
Goats	**13 variants (+ null alleles)** (Bevilacqua et al., 2002)	6 variants (+1 null allele) (Sacchi et al., 2005)	3 variants (+ null alleles)	**13 variants** (Prinzenberg et al., 2005)	No variants characterised so far (Ballester et al., 2005)	–
Sheep	5 variants, A to E (Chianese et al., 1996)	3 variants, A to C (Chianese et al., 1996)	2 variants (Ceriotti et al., 2004)	2 variants (Ceriotti et al., 2004)	3 variants, A to C	2 variants, A & B

Data taken from Ng-Kwai-Hang and Grosclaude (2003)

proteomics (mass spectrometry) have particularly highlighted how such genomic rearrangement contributes to changes in the milk protein gene complement of mammals and how genetic polymorphisms are responsible for the extreme complexity and the large variability (qualitative and quantitative) of the milk protein fraction, between, but also within, species. High conservation of MFGM protein-encoding genes between monotremes' and placental mammals' genomes strongly suggests that they are crucial for lipid secretion and that the secretory function was already established 150 million years ago (Lemay et al., 2009).

Our purpose here is to provide the reader with an overview of the current knowledge of milk protein variability between species, both at the structural (amino acid sequence, post-translational modifications (PTM)) and the quantitative (ultimately absence) levels. Genetic polymorphisms, when responsible for deep modifications, will also be considered.

13.2 Caseins

Caseins are phosphoproteins synthesised by the MEC under multihormonal control as more or less large and stable particles, referred to as casein micelles and which appear like raspberries in electron micrographs (Dalgleish et al., 2004). These spherical particles might be the result of aggregation of smaller discrete subunits or submicelles (Schmidt 1982; Walstra 1990) cemented by a calcium phosphate salt (colloidal calcium phosphate). However, this casein micelle model remains a topic of discussion and controversial debates (McMahon and Oommen 2008). Casein micelles are present in the milk of all mammals and have a statistically broad distribution in size, ranging between 60 and 600 nm (Holt 1985, 1992). In bovine milk, still the most thoroughly studied milk to date, casein micelles are made of several casein molecules (Schmidt 1982) cemented by a calcium phosphate salt. These proteins arise from the expression of four single-copy autosomal genes which encode four distinct polypeptide chains (α_{s1}-, β-, α_{s2}- and κ-caseins).

These 4 genes (5 in some species, see below), of which the structures are now known in several species (Rijnkels et al., 2003), are clustered (physically linked), in this order, on the same chromosome (chromosome 6 in cattle and goat, 5 in mouse and 4 in human), whatever the species. Differences reported between species will be discussed briefly, since such genomic data provide clues that can probably improve our understanding of the mechanisms responsible for specific variations in the number and relative proportions of caseins as well as in their total concentration across mammalian milks. Caseins amount to nearly 80% (i.e. 25–28 g/L) of the whole protein fraction in the milk of ruminants, whereas in human milk, the casein percentage is lower, not exceeding 50% (i.e. 5–8 g/L). Conversely, the casein content of some lagomorphs' milk can reach 200 g/L (Fig. 13.1).

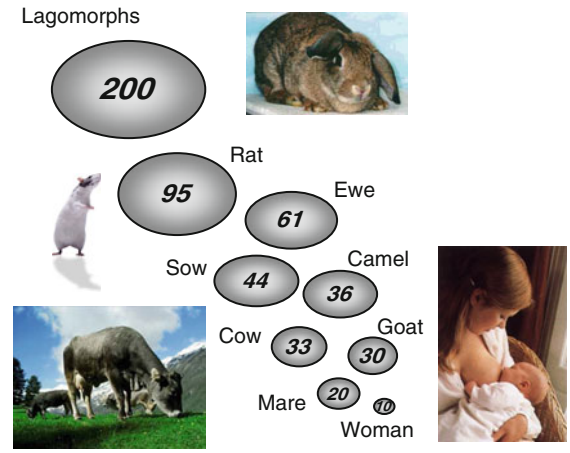


Fig. 13.1 Concentration (g/L) of milk proteins in eight species (from Jenness 1974; Holt and Jenness 1987; Grabowski et al., 1991; Martin 1996)

Besides this wide quantitative variability, it is worth noting that, between species, a high rate of sequence divergence generally occurs in orthologous gene products. Hence, the casein fraction of milk is a complex and specific system which deserves to be considered in terms of diversity, particularly in light of the growing number of biologically active peptides derived from milk proteins, including caseins, identified during the last 20 years (Clare and Swaisgood 2000; Meisel 2005).

In addition, post-translational processing, such as phosphorylation, glycosylation and limited proteolysis by plasmin, increases the heterogeneity of this system which is complicated even more by the occurrence of genetic variants. The primary focus of this chapter will be interspecies comparisons in terms of quantitative and structural variability. Nevertheless, within-species variability will be also considered when it is a species-specific feature. Particular attention will be paid to discrete phosphorylation and exon-skipping events which contribute to protein diversity and evolution and very likely to specific micellar organisation.

13.2.1 The Casein Gene Locus (*CSN*) and Quantitative Variability

Caseins are present in the milk of all mammals. However, their total concentration and their relative proportions are largely species dependent. The species studied so far produce more or less

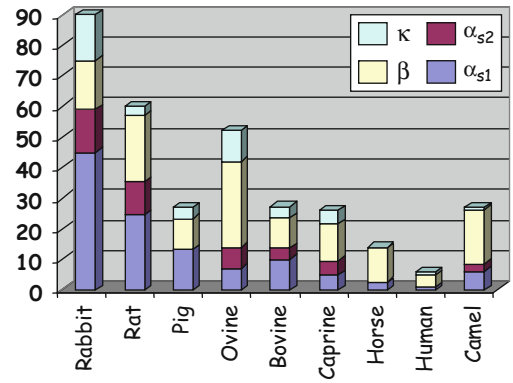


Fig. 13.2 Comparative properties of milk caseins for nine species. Numbers correspond to mean values from several data sets in the literature (Grabowski et al., 1991; Ribadeau Dumas and Brignon 1993; Martin and Grosclaude 1993; Kappeler et al., 1998; Ginger and Grigor 1999; Miranda et al., 2004). The percentage of α_{s1}-casein given for sow's milk corresponds to α_s- caseins ($\alpha_{s1} + \alpha_{s2}$)

large quantities of milks whose protein content ranges between 10 and 200 g/kg (Fig. 13.1). Human milk has one of the lowest protein content (10 g/kg), whereas that of rabbit milk is undoubtedly one of the highest (200 g/kg). Amongst dairy ruminants, with more than 50 g/kg, sheep milk has the highest total protein content. Beyond this large variability in the milk protein content, there are large differences in the relative proportions of caseins, between species (Fig. 13.2). Thus, in human milk, β-casein is, by far, the main casein component. Conversely, α_{s1}-casein predominates in rabbit milk. The milk of

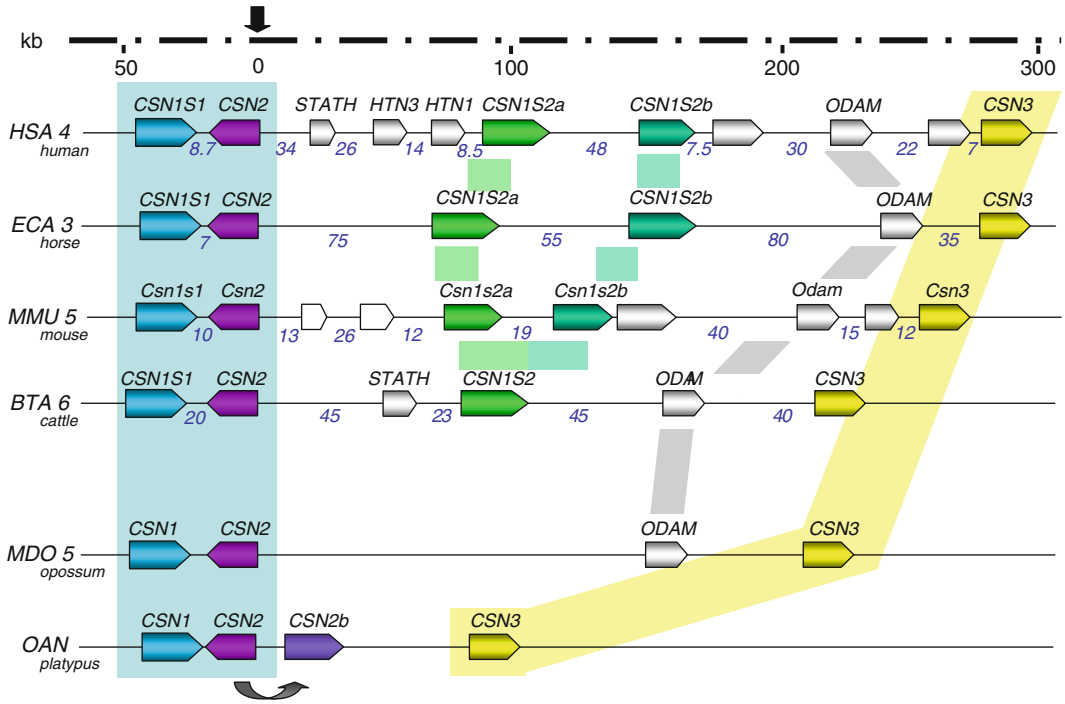


Fig. 13.3 Evolution of the casein locus organisation. The casein loci from platypus (*Ornithorhynchus anatinus*), opossum (*Monodelphis domestica*), cattle (*Bos taurus*), mouse (*Mus musculus*), horse (*Equus caballus*) and human (*Homo sapiens*) genomes are drawn approximately to scale in order to underline the expansion of this locus during the course of mammalian evolution (adapted from Lefèvre et al., 2009; Warren et al., 2008, taking into account additional genomic information from the NCBI). Genes are depicted by *arrow boxes*, giving the orientation of transcription. *Empty boxes* represent putative genes based on similarity, of which the expression remains to be demonstrated. Intergenic region sizes are given in kb. The human and horse loci have approximately the same size (320/330 kb), whereas the cattle locus (250 kb) is *ca.* 80 kb shorter. Whilst the marsupial (opossum) locus is close in size to the cattle locus, on the other hand, the monotreme (platypus) locus is significantly smaller (less than 150 kb) with a duplication of *CSN2* (*grey arrow*), which occurred recently in this lineage. *STATH* (statherin) and *HTN3* and *HTN1* (histatins) are genes having a common origin and encoding salivary proteins that protect teeth by regulating the spontaneous precipitation of calcium phosphate salts on enamel surface (Kawasaki and Weiss 2003). *ODAM* is a gene highly conserved across species encoding the odontogenic ameloblast-associated protein, a tooth-associated epithelia protein that probably plays a role in odontogenesis, possibly incorporated into the enamel matrix at the end of the mineralisation process (Kestler et al., 2008), but also conspicuous by its expression in several epithelial tissues (Moffatt et al., 2008). *CSN1S1*, *CNS1S2*, *CSN2*, and *CSN3* are genes encoding α_{s1}-, α_{s2}-, β- and κ-casein, respectively

rat, porcine and bovine contains approximately the same proportion of β- and α_{s1}-caseins. Today, human milk remains the only thoroughly studied milk in which α_{s2}-casein has not been found. The presence of two different α_{s2}-caseins has been detected recently in equidae milk (Martin et al., unpublished).

Comparative analysis of the casein locus organisation (Fig. 13.3) appears to be highly conserved between species, even for ancestral mammals such as monotremes (platypus) in which casein genes are tightly clustered together in the genome, as they are in placental mammals (Warren et al., 2008). The genomic organisation of the platypus casein locus has been elucidated and compared with other mammalian genomes, including the marsupial opossum and several eutherians (Lefèvre et al., 2009). Whereas the physical linkage of casein genes has been confirmed in platypus, a recent duplication of *CSN2* was observed in the monotreme lineage, as opposed to more ancient duplications of *CSN1S2* in the eutherian lineage, whilst marsupials possess only single copies of α- and β-casein-encoding genes. Another striking

feature is the close proximity between *CSN1* and the main *CSN2*. The lineage-specific gene duplications that have occurred within the casein locus of monotremes and eutherians but not marsupials, which may have lost part of the ancestral casein locus, emphasise the independent selection on milk provision strategies to the young, most likely linked to different developmental strategies (Lefèvre et al., 2009).

The four (or five) genes are confined to a 250–350 kb region on chromosome 6 in cattle and goats (Threadgill and Womack 1990; Hayes et al., 1993) and arranged in the order α_{s1}, β, α_{s2} and κ. In the goat, loci encoding α_{s1}- and β-caseins were shown to be *ca.* 12 kb apart and convergently transcribed (Leroux and Martin, 1996). These results were confirmed in cattle for which the genomic organisation of the casein gene locus was determined (Rijnkels et al., 2003). Despite some differences in the distance separating casein genes and their numbers, the overall organisation of the locus is fairly well conserved, and the presence of dominant *cis*-acting regulatory elements, required for the high-level coordinate expression of the casein genes, is suspected in the α_{s1}-/β-region (Rijnkels et al., 1997).

Indeed, all four genes are coordinately expressed at high levels in a tissue- and stage-specific fashion. The three genes encoding the "calcium-sensitive" caseins (α_{s1}, α_{s2} and β), that are related through evolution, share common regulatory motifs in the proximal 5′-flanking region (Groenen et al., 1993). Although the organisation of the 5′-flanking region of the κ-casein gene is different (Coll et al., 1995), its expression pattern seems to be similar to that of the other casein genes. There is more and more evidence demonstrating that a common set of transcription factors is required in most mammalian species for the expression of milk protein genes. The mechanisms controlling milk protein gene expression, especially pertaining to the behaviour of Stat5, in the cow are significantly different from the mouse (Wheeler et al., 1997). More precisely, the different organisation of the hormone response regions of casein genes, from the binding of factors such as Stat5 and C/EBP, in different mammalian species, apparently does not result in fundamental differences in their responsiveness to lactogenic hormones, at least in transfected cell lines. The species-specific arrangement of transcription factor binding sites in the β-casein gene appears to be crucial for the strength and stage at which this gene is expressed in different species, including human, rodents and ruminants (Winklehner-Jennewein et al., 1998). For example, the bovine, but not the mouse, β-casein gene is strongly induced shortly before parturition. This difference in stage-specific expression was recapitulated in the expression of a bovine β-casein transgene (including 16 kb of 5′- and 8 kb of 3′-flanking regions) in transgenic mice, thus indicating that *cis*-acting sequences might be, at least in part, responsible for species-specific expression patterns (Rijnkels et al., 1995).

Nevertheless, transcription is not the only level at which regulation of gene expression may occur. In the following, we will see that there are many other factors acting at the post-transcriptional level, including messenger RNA stability and processing, as well as translational regulation (Bevilacqua et al., 2006; Rhoads and Grudzien-Nogalska, 2007). The protein-coding regions of most vertebrate genes, including those encoding caseins in mammals, are split. Most eukaryotic messenger RNAs are thus transcribed as precursors (pre-messengers) containing intervening sequences (introns) which have to be removed to generate mature and functional mRNAs. The process of intron removal, and exon joining (splicing), is a major function ensured, in the nucleus, by a large multicomponent (five small nuclear RNAs and more than 50 proteins) complex, called spliceosome, assembled in a stepwise pathway. This accurate mechanism is governed by a set of rather strict rules to achieve high fidelity and efficiency in splicing. However, caseins spliced variants are widely spread across species. A dysfunction of this machinery may have dramatic biological consequences by modifying the message and accordingly the primary structure of the protein. This is well exemplified in mare's milk in which a low-molecular-weight β-casein variant, showing a 132 amino acid residues internal deletion, has been characterised as arising from a cryptic splice site usage occurring

within exon 7, during the course of primary transcripts processing (Miclo et al., 2007). Such deviant splicing behaviour might be regulated by an intronic splicing enhancer, sometimes located far away from the splicing site, as was shown for the gene encoding β-casein in mare mammary gland (Lenasi et al., 2006).

13.2.2 Primary Structure of Caseins: Comparison Across Species

Since the elucidation of the primary structure of bovine α_{s1}-casein by Mercier et al. (1971), the complete amino acid or nucleotide (cDNA, gene) sequence of the four (five) caseins has been determined in a number of species (including human, horse, ruminants and rodents). Multiple alignments, which help to define functional domains, performed with sequences available today, can reach highly informative levels, provided that the structural intron/exon organisation of the gene is taken into account. Indeed, in such a way, multiple alignments, in which gaps were introduced to maximise the alignment, reveal the conserved regions but also highlight their evolutionary pathways. This is particularly true for the α_s-caseins, the genes for which comprise up to about 20 exons.

13.2.2.1 α_{s1}-Casein

A multiple alignment of α_{s1}-casein from 12 species is presented in Fig. 13.4. Even tuning the alignment, taking into account the exon modular splitting derived from known gene structural organisations (Koczan et al., 1991; Leroux et al., 1992; Jolivet et al., 1992), there are few even short segments of amino acid identity across the 12 species. Conversely, such a method of alignment immediately indicates the occurrence of insertion/deletion events. Exon skipping, first found in goat α_{s1}-casein (Leroux et al., 1992) and in human β-casein (Menon et al., 1992a, b; Martin and Leroux, 1992), was shown to be responsible for such events and for the apparent relatively high structural divergence observed between α_{s1}-caseins from different species, as well as for its wide variability in size. α_{s1}-Casein ranges from 183 to 199 amino acid residues, in guinea pig and cattle, respectively, whereas, due to the insertion of a tandem repeated hexapeptide sequence (QASLAQ), the protein is significantly larger (from 280 to about 300 amino acid residues) in mouse (Hennighausen et al., 1982) and rat (Hobbs and Rosen, 1982). This sequence was shown to correspond to a short "virtual exon" occurring within intron 13 of the bovine gene and surrounded by quite perfect consensus splice sequences (Martin et al., 1996). In addition, the same short sequence is recognised as an exon in the porcine α_{s1}-casein mRNA (Alexander et al., 1992).

The three hydrophobic domains identified in the bovine molecule, spanning residues 1–44, 90–113 and 132–199, are more or less well conserved between species. The most highly conserved region, except the signal peptide, remains, however, the multiple phosphorylation site, encoded by the 3′ end of exon 9. This SerP cluster is confined within a sequence (encoded by exons 7–10) carrying a high net negative charge (7 SerP, 3 Asp and 8 Glu, in bovine), at the natural pH of milk, whilst the remainder of the molecule is, under such conditions, essentially uncharged. These features are rather well conserved in the 12 compared species, exemplified herein by the third hydrophobic domain (residues 132–199), corresponding to the seventeenth exon, probably being one of the most conserved parts of the molecule. α_{s1}-Casein does not contain any cysteine or cystine, except in rodents (Hobbs and Rosen, 1982; Grusby et al., 1990) and humans (Rasmussen et al., 1995; Johnsen et al., 1995; Martin et al., 1996), a feature which is usually found in α_{s2}-casein. In this connection, it is worth noting that, at least in human milk, in which the presence of α_{s2}-casein still remains to be demonstrated (Rijnkels et al., 2003), α_{s1}-casein is capable of forming disulphide-linked heteromultimers with κ-casein which contains only one cysteinyl residue (Rasmussen et al., 1999).

13.2.2.2 β-Casein

With 209–217 residues, in cattle and pig, respectively, without any cysteinyl residue, this casein is the most hydrophobic of the four caseins. It is especially rich in proline and displays, in all species, an amphipathic structure with a single multiple phosphorylation

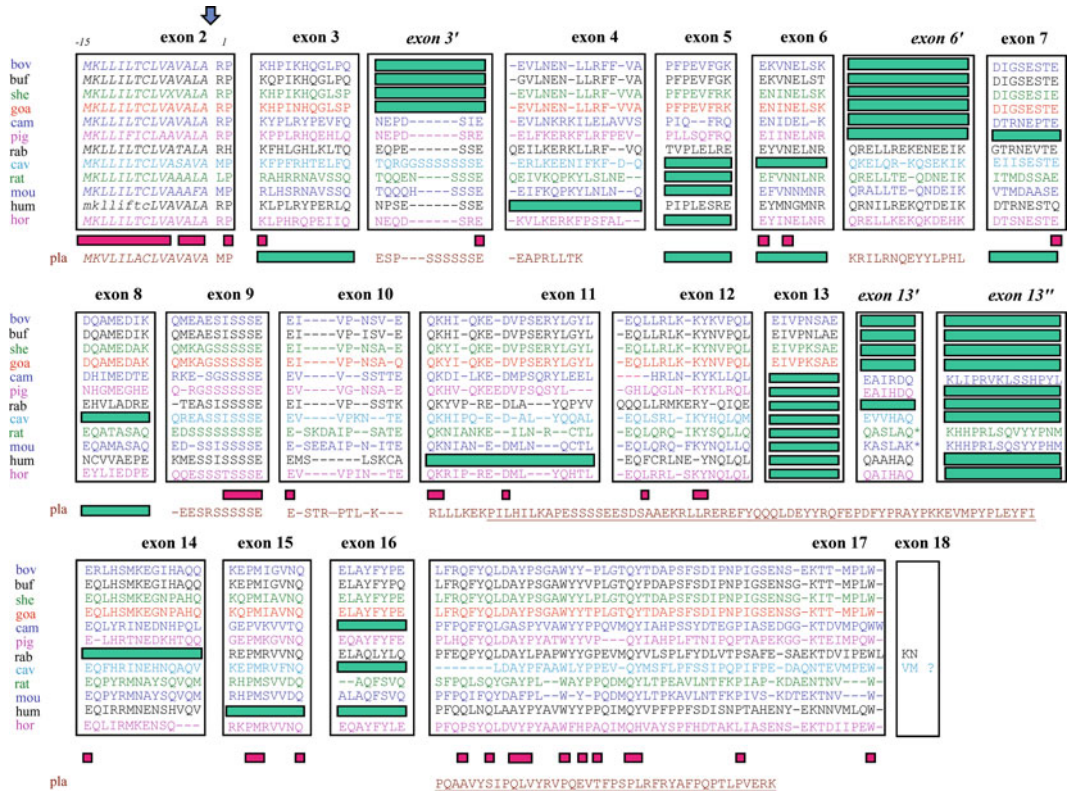


Fig. 13.4 Multiple alignment of the amino acid sequence of α_{s1}-casein from 12 eutherian species. Abbreviations and accession numbers are given in parentheses: cow (bov, M38641), water buffalo (buf, AJ005430), sheep (she, X03237), goat (goa, X59836), camel (cam, AJ012628), pig (pig, X54973), rabbit (rab, X13042), guinea pig (cav, X00938), rat (rat, J00710), mouse (mou, M36780), horse (hor, NM_001081883) and human (hum, X98084). Peptide sequences are split into blocks of amino acid residues to visualise the exonic modular structure of the protein as deduced from known splice junctions of the bovine (Koczan et al., 1991), goat (Leroux et al., 1992) and rabbit (Jolivet et al., 1992) genes. Exon numbering (*in bold*) is that of the ruminants genes. Additional exons are numbered in *single quotes* and *double quotes* (*in italics*). *Large green boxes* within blocks depict species-specific constitutively outspliced exons. *Red boxes* under exonic blocks identify highly conserved amino acid residues (>9/12) between species. Guinea pig α_{s1}-casein is the sequence of casein B characterised by Hall et al. (1984b). *Italics* correspond to the signal peptides, of which the cleavage site is indicated by the *vertical blue arrow*. *Spaced dashes* are inserted gaps introduced to maximise the alignment. *Asterisk* refers to the basic motifs of tandem hexapeptide repeats occurring in the rat and mouse α_{s1}-caseins. A monotreme (platypus: pla) CSN1 casein sequence (FJ548613) is also given for comparison. *Underlined* sequence represents part of the protein falling in an unresolved platypus genome sequence (Lefèvre et al., 2009)

site, located in the N-terminal part of the molecule. At the pH of milk, the N-terminal sequence (30 residues) is highly negatively charged (an average of 11 negative charges, 7 Glu and 4 SerP, in ruminants concentrated in the 21 first N-terminal amino acid residues), whereas the rest of the peptide chain, which is highly hydrophobic, has no net charge. Such a feature explains the property of β-casein which allows for micellar aggregates to be formed in solution.

Eleven of the same 12 species, except the guinea pig, of which the β-casein sequence is not available, are compared and aligned in Fig. 13.5. There is no evidence in the extensive literature related to guinea pig caseins for the existence of β-casein. However, there is no irrefutable evidence for its absence from guinea pig milk. Again, as previously mentioned for α_{s1}-caseins, the conservation of the leader peptide is strikingly notable. Moreover, with *ca.* 80% homology

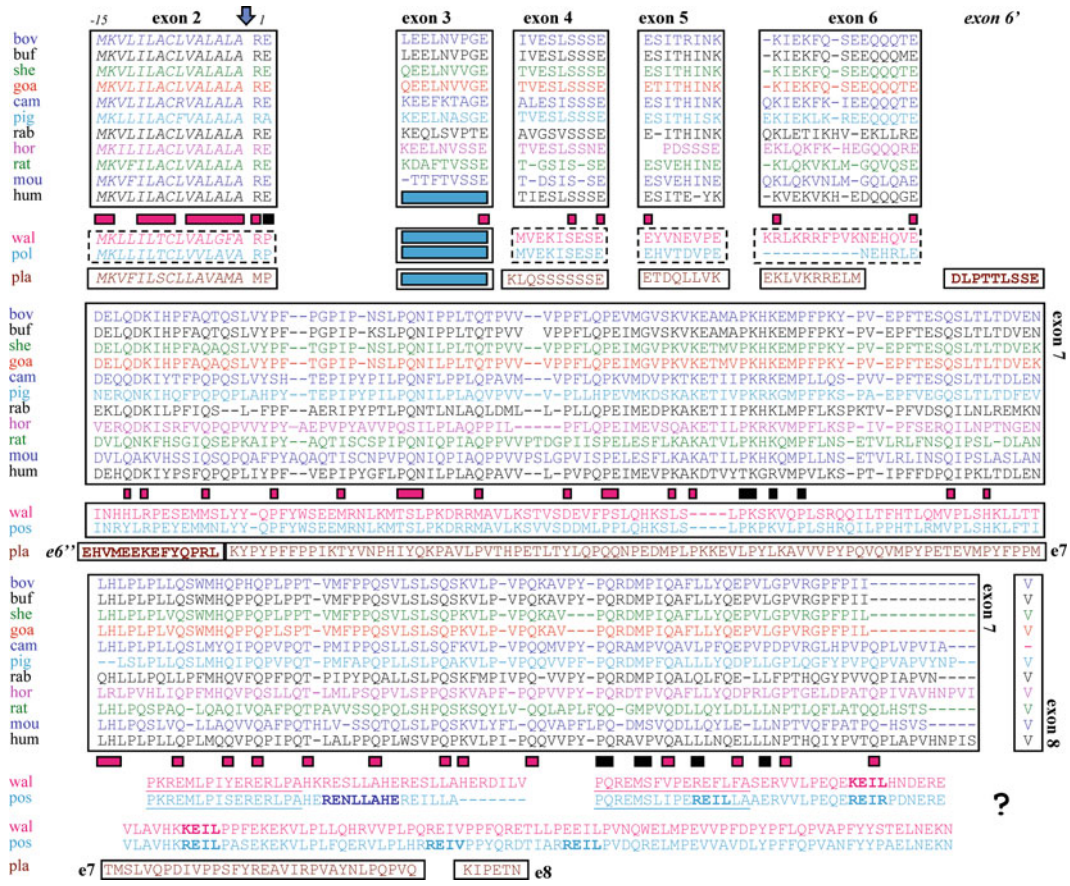


Fig. 13.5 Multiple alignment of the amino acid sequence of β-casein from 11 eutherians. Abbreviations and accession numbers are given in parentheses: cow (bov, M15132), water buffalo (buf, AJ005165), sheep (she, X16482), goat (goa, AH001195), camel (cam, AJ012630), pig (pig, X54974), rabbit (rab, X13043), rat (rat, J00711), mouse (mou, X04490), human (hum, X17070) and horse (hor, NM_001081852, Q9GKK3 on Expasy UniProtKB). Two marsupial, tammar wallaby (wal, X54715) and possum (pos, AF128397), as well as a monotreme (platypus: pla, FJ548612) sequences are also given. Peptide sequences are split into blocks of amino acid residues to visualise the exonic modular structure of the protein as deduced from known splice junctions of the rat (Jones et al., 1985), the bovine (Bonsing et al., 1988), sheep (Provot et al., 1995), the rabbit (Thépot et al., 1991) and the human (Hansson et al., 1994) genes. Exon numbering (*in bold*) is that of ruminants genes. Additional exons are numbered in *single quotes* and *double quotes* (*in italics*) for platypus sequence. *Large blue boxes*, within blocks depict species-specific constitutively outspliced exons. *Red* and *black boxes*, between eutherian and marsupial sequences, identify highly conserved amino acid residues (>10/14) between species and anchoring points of marsupial sequences, respectively. *Italics* correspond to the signal peptides, of which the cleavage site is indicated by the *vertical blue arrow*. *Spaced dashes* are inserted gaps introduced to maximise the alignment. *Underlined* and *bold* amino acids in the marsupial sequences depict duplications and the basic motif of the tandem octapeptide repeats found in marsupials, respectively

between α_{s1}- and β-casein signal peptides, the evolutionary relationship between the genes encoding calcium-sensitive caseins is further substantiated. The close proximity of the main α- and β-casein proteins in monotremes strongly supports this statement. On the other hand, at the level of the mature proteins, the poly-phosphorylated region (encoded by exon 4) is no longer the only region showing a clear conservation between species. Indeed, all along the large and mainly hydrophobic sequence, encoded by exon 7 (more than 160 amino acid residues), a rather high level

of homology (30%) is observed. This ratio is quite good, given the number of sequences aligned, having a number of isolated amino acid residues (Q, P, K and L) conserved. Furthermore, most of substitutions tend to be conservative (Holt and Sawyer, 1988).

Albeit less frequently, probably owing to its less split genomic organisation, exon skipping also occurs during the course of the processing of primary transcripts from the β-casein gene, in humans (Menon et al., 1992a, b; Martin and Leroux 1992) and horse (Miranda et al., 2004; Lenasi et al., 2006; Miclo et al., 2007). Despite apparent high dissimilarity with those of eutherian species, two marsupial (tammar wallaby and brushtail possum) sequences (Collet et al., 1992; Ginger et al., 1999) and one monotreme (platypus) sequence (Lefèvre et al., 2009) have been included in the alignment (Fig. 13.5). Interestingly, this attempt strongly suggests again an outsplicing of exon 3, as reported for humans. Elsewhere, tammar β-casein and that isolated from the milk of the common brush-tailed possum, which are by far larger than the others (270 amino acid residues for the mature polypeptide chain vs. 209 in cattle), display a tandemly repeated (16 or 17 times) octapeptide sequence (RESLLAHE) in the C-terminal part of the molecule. This strongly supports the notion that the gene encoding β-casein might have grown through intragenic duplication, eventually coupled with (or followed by) changes of splice sites, before being subjected subsequently to duplications, giving rise to the cognate genes that have then evolved divergently.

Considered together, these observations support the hypothesis (Jones et al., 1985) of a presumed primitive and common ancestral gene resulting from the recruitment into a functional gene with a minimum of five exons: the first and the last corresponding to the 5′ and 3′ non-coding regions; the second encoding the signal peptide; the third, a highly hydrophilic region including a multiple phosphorylation site; and the penultimate coding for a hydrophobic sequence required to ensure aggregation properties, which in turn is essential for casein micelle formation. Sequence similarities between the first exons of the genes encoding the 3 calcium-sensitive caseins confirm such an assumption.

13.2.2.3 α_{s2}-Casein

α_{s2}-Casein was the last bovine casein to be sequenced (Brignon et al., 1977). Of the calcium-sensitive caseins, it is the most highly phosphorylated. α_{s2}-Casein occurs in milk in several forms and differs in the level of phosphorylation (10–13 phosphate groups/molecule). The peptide chain is 207 amino acid residues long, and the phosphate groups are clustered in three regions of the molecule (7–31, 55–66 and 129–143). Peptide segments spanning residues 68–125 and the C-terminal part of the protein are predominantly hydrophobic (Holt and Sawyer 1988). Given that human milk does not appear to contain α_{s2}-like casein and the equine sequence is still not available, amino acid sequence comparisons are restricted to only 10 eutherian species (Fig. 13.6).

The structural organisation of the α_{s2}-casein gene, first determined for the bovine species (Groenen et al., 1993), provides evidence that genes encoding α_{s2}- and β-caseins are more closely related to each other than to the α_{s1}-casein gene. However, analyses of interspecies relationships performed at the transcript level, show that α_{s2}-caseins have diverged through extensive sequence rearrangements and a high level of nucleotide substitution (Stewart et al., 1987). A tandem repeat was first detected in the amino acid sequence of bovine α_{s2}-casein (Brignon et al., 1977). On the basis of the gene sequence (Groenen et al., 1993), the large internal repeat was precisely extended to codons 43–124 and 125–204 which resulted in the formation of exons 12–16 by a duplication of exons 7–11. In addition, from both amino acid and nucleotide sequence comparisons, it is still evident that, with up to 60% similarity, exons 3–5 also arise from a duplication event of exons 8–10. Therefore, it can be hypothesised that this gene has been subjected to two successive duplications of a 5-exons module followed by the loss of one upstream (exon 7/12) and one downstream (exon 11/16) exon.

The same observation can be made for the other artiodactyls, the camel, the rat and the guinea pig sequences, whilst for the mouse and the rabbit, the

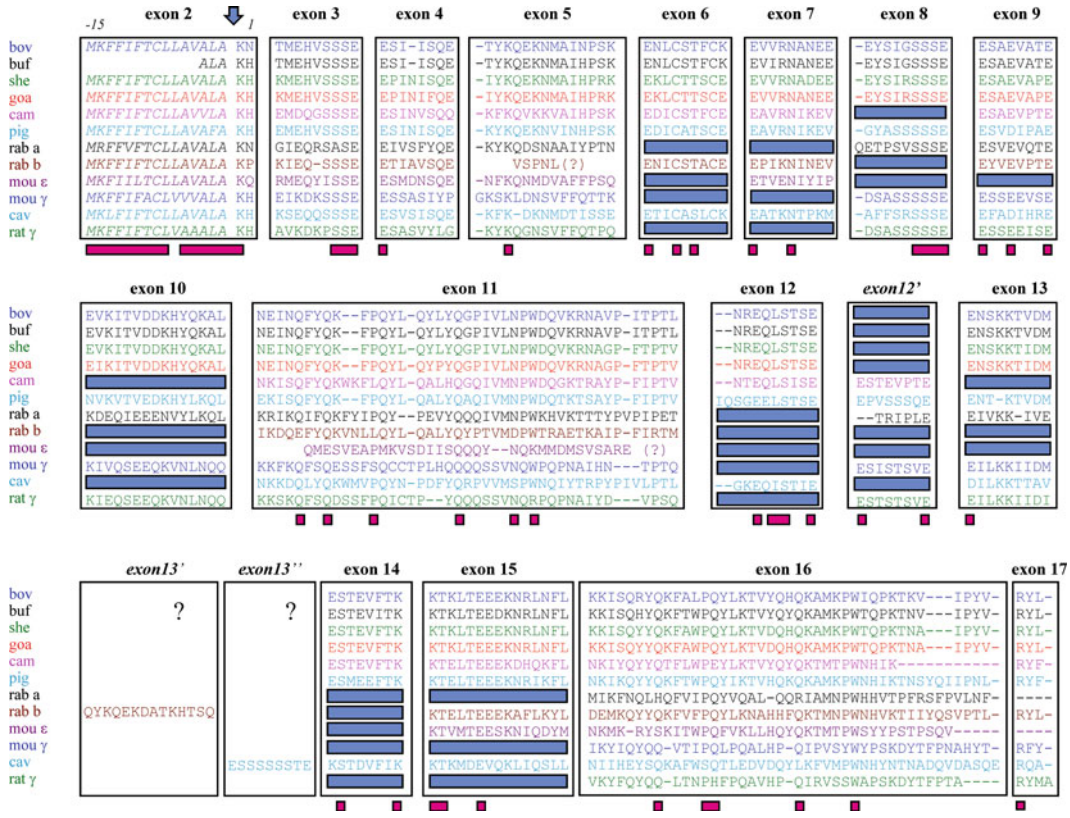


Fig. 13.6 Multiple alignment of the amino acid sequence of α_{s2}-casein from ten mammalian species. Abbreviations and accession numbers are given in parentheses: cow (bov, M16644), water buffalo (buf, AJ005431), sheep (she, X03238), goat (goa, X65160), camel (cam, AJ012629), pig (pig, X54975), rabbit (rab a, X76907; rab b, X76909), mouse (mou ε, J00379; mou γ, D10215), guinea pig (cav, X00374) and rat (rat γ, J00712). Two sequences are given for rabbit (a and b) and mouse (δ/ε and γ). The sequence for the rat and the guinea pig corresponds to the γ-casein (Hobbs et al., 1982) and to casein A (Hall et al., 1984a), respectively. Peptide sequences are split into blocks of amino acid residues to visualise the exonic modular structure of the protein, as deduced from known splice junctions of the bovine gene (Groenen et al., 1995). Additional exons are numbered in *single quotes* and *double quotes* (*in italics*). *Large blue boxes*, within blocks depict species-specific constitutively outspliced exons. *Red boxes* identify highly conserved amino acid residues (>10/12) between sequences. *Italics* correspond to the signal peptides, of which the cleavage site is indicated by the *vertical blue arrow*. *Spaced dashes* are inserted gaps introduced to maximise the alignment

situation is not so clear and complicated by the existence of two (ε/δ and γ or α_{s2}-a and b, respectively) α_{s2}-casein genes (Hennighausen et al., 1982; Sasaki et al., 1993; Dawson et al., 1993). In these species, the α_{s2}-like caseins are shorter than the others since they are 143, 184, 180 and 182 amino acid residues long, respectively, and all the duplicated sequences were apparently lost in mouse ε/δ-casein. Thus, there are marked differences in size. Here too, deleted segments, emphasised by multiple alignments based on the structure of the bovine gene, correspond to exon sequences outspliced from the mature messengers during the processing of the primary transcripts. Camel α_{s2}-casein, with 178 amino acid residues, lacks two internal stretches of 9 and 15 amino acid residues, very likely corresponding to exons 8 and 10. It now remains to demonstrate the presence, at the genomic level, of the relevant nucleotide sequence (encoding the missing peptide segment) and to characterise mutations responsible for this double exon-skipping event, as had been done for the

bovine α_{s2}-casein D variant (Bouniol et al., 1993). On the other hand, as previously mentioned for α_{s1}-casein, additional exons may also break out from intron sequences. The presence of an extra peptide sequence (IQSGEELST), between exon 11 and 12 in pig α_{s2}-casein, could be due to this kind of event. Similarly, one can anticipate the probable existence of an additional exon sequence within intron 14 of the gene encoding α_{s2}-casein b in the rabbit genome.

One of the main biochemical features of α_{s2}-casein, usually underlined and plausibly having an important functional role, is its ability to form disulphide bridges, owing to the presence of two cysteinyl residues at positions 36 and 40, in the mature bovine peptide chain. These residues are encoded by exon 6, the sequence of which is rather well conserved, when present, with both cysteinyl residues at the same position. In contrast, in rat (γ), mouse (ε/δ and γ) and rabbit α_{s2}-casein, which lack this exon, both cysteinyl residues are obviously removed. Nevertheless, with a single and two contiguous cysteinyl residues in the middle of the peptide sequence encoded by exon 11 in rat and mouse γ-caseins, respectively, and since rat, mouse and human α_{s1}-caseins contain at least one cysteinyl residue, there is no eutherian milk in which any α_s-casein is devoid of a cysteinyl residue.

13.2.2.4 κ-Casein

Glycosylated at various levels, κ-casein is highly heterogeneous, soluble in presence of calcium and differs considerably in structure from the calcium-sensitive caseins. The functional duality of κ-casein, which is to interact hydrophobically with the other caseins and at the same time provide a hydrophilic and negatively charged surface on the micelle to stabilise the colloidal suspension, is strikingly reflected by its amphipathic primary structure. Its hydrophilic and flexible C-terminal part (caseinomacropeptide or CMP) is cleaved specifically by chymosin (between residues Phe_{105} and Met_{106}, in ruminants), thus leading to the destabilisation of the micelle, to which the highly hydrophobic and insoluble N-terminal part (*para*-κ-casein) remains anchored. Two cysteine residues are found in the *para*-κ-casein region.

Although it belongs to the same chromosomal casein locus, the gene encoding κ-casein does not share any common structural organisation scheme with the other casein genes. It is thought to be evolutionarily related to the γ fibrinogen gene (Jollès et al., 1978; Alexander et al., 1988), which encodes a protein similarly involved in a clotting process (blood), following a limited proteolytic cleavage.

Interspecies comparison reveals that the κ-casein gene is identically organised in ruminants (Alexander et al., 1988; Persuy et al., 1995), rabbit (Baranyi et al., 1996) and humans (Edlund et al., 1996). The transcription unit invariably comprises 5 exons, three of which are small (65, 62 and 33 nucleotides in bovine), encoding the 5′-untranslated region (5′-UTR) (exon 1) and the signal peptide (exons 2 and 3) which is longer (21 vs. 15 residues in the calcium-sensitive caseins). Therefore, the majority of the mature protein sequence (160 amino acid residues) is encoded by exon 4, whilst the last exon encodes the 3′-untranslated region.

Multiple alignments from the same 12 species, considered in this chapter, are shown in Fig. 13.7. Gaps have been introduced to maximise similarity, taking into account comparisons performed at the nucleotide level of exon 4 (Cronin et al., 1996; Gatesy et al., 1996) for higher ruminants as well as 21 other species including cetaceans, hippo, deer, giraffe, tapir and zebra.

13.2.3 Molecular Diversity of Caseins: Interspecies Variability

In addition to differences in primary structure across species, examined above, which reflect changes at the genomic level within coding sequences and/or flanking sequences (splice site consensus sequences), further sources of variation occur at a post-transcriptional level. They affect mainly the processing of the primary transcripts and PTM such as phosphorylation (all caseins) and glycosylation (κ-casein). The extent of this variability and the complexity of the specific pattern within each species provide further criteria of distinctiveness between species.

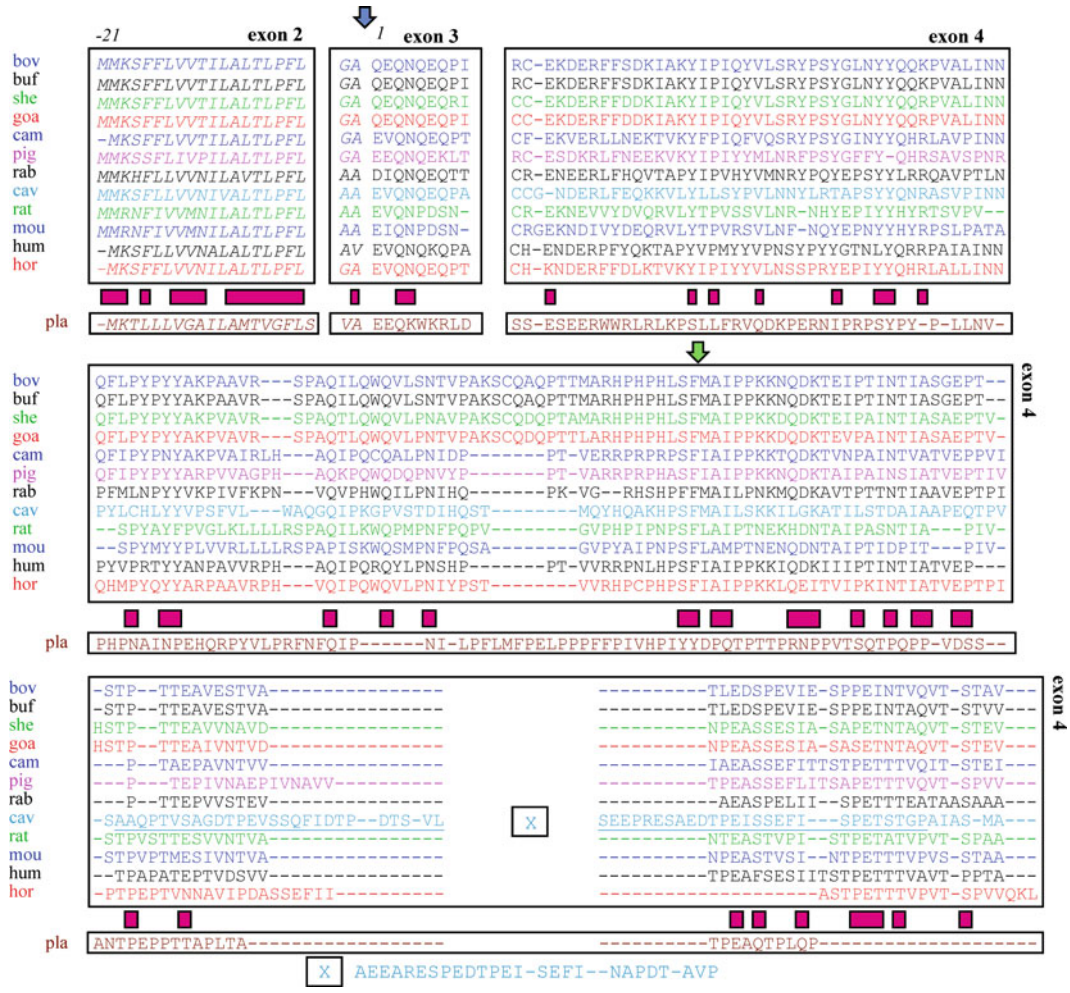


Fig. 13.7 Multiple alignment of the amino acid sequence of κ-casein from 12 mammalian species. Abbreviations and accession numbers are given in parentheses: cow (bov, M36641), water buffalo (buf, AJ011387), sheep (she, X51822), goat (goa, X60763), camel (cam, Y10082), pig (pig, X51977), rabbit (rab, Z18243), guinea pig (cav, X56020), rat (rat, K02598), mouse (mou, M10114), horse (hor, NM_001081884 (NCBI) and P82187 (UniProtKB, Expasy)) and human (hum, M73628). Peptide sequences are split into blocks of amino acid residues to visualise the exonic modular structure of the protein, as deduced from known splice junctions of the bovine (Alexander et al., 1988), the rabbit (Baranyi et al., 1996) and the human (Edlund et al., 1996) genes. *Red boxes* identify highly conserved amino acid residues (>10/12) between species. *Italics* correspond to the signal peptides, of which the cleavage site is indicated by the *vertical blue arrow*. *Spaced dashes* are inserted gaps introduced to maximise the alignment. The chymosin-sensitive bond is indicated by the *green vertical arrow*. X corresponds to the basic motif of the repeated sequence (*underlined*) in the guinea pig. The platypus (pla, FJ548626) sequence is also given, taking into account the structural organisation of the gene (Lefèvre et al., 2009)

13.2.3.1 Defects in the Processing of Primary Transcripts: Splice Variants

Two kinds of events may arise during the processing of the primary transcripts, both leading to a shortening of the peptide chain length. The first event, referred to as cryptic splice site usage, primarily due to a "slippage" of the splicing machinery, is induced by a "favourable" junction sequence. As far as caseins are concerned, this defect in accuracy leads to the loss of the first codon (usually a CAG) of the 3′ exon. The second event, which is particularly well exemplified by small ruminants, gives rise to a casual alternative exon

skipping (sometimes referred to as non-allelic exon skipping). It is thought to be caused by weaknesses in the consensus sequences, either at the 5′ and/or 3′ splice junctions or at the branch point, or both.

Casual Usage of Cryptic Splice Sites

The casual deletion of a glutaminyl residue (Gln_{78} or Q_{78}), first detected in goat α_{s1}-casein (Leroux et al., 1992), seems to be a rather frequent phenomenon, occurring in most of the species examined so far. This codon skipping, which is likely due to an erroneous 3′ cryptic splice site usage when exons 10 and 11 are joined, has been found in the four major ruminant species (Ferranti et al., 1997, 1999). It is worth noting that this kind of event may play a functional role in the structure and stability of casein micelles, since Gln_{78} is located at the junction between the polar cluster of phosphoseryl residues and the hydrophobic domain of the protein. Interestingly, such a cryptic splice site event occurs not only in ruminants. In the human, α_{s1}-casein transcript, in which exon 11 is lacking, a glutaminyl residue (Q_{37} in the mature protein), encoded by the first codon of exon 6′, an additional exon found in the intron-bridging exon 6 to exon 7 (Martin and Leroux, unpublished results), was also shown to be casually absent (Johnsen et al., 1995; Martin et al., 1996).

Examples of glutaminyl residue insertion/deletion in protein, due to cryptic splice site usage, are well-documented (Condorelli et al., 1994; Vogan et al., 1996; Hayashi et al., 1997), and they have been shown recently to occur also with other calcium-sensitive casein pre-messengers and for species other than humans and ruminants. Boumahrou et al. (2011) reported in mice the loss of the first codon (CAG) in exon 6 and 8[1] of *Csn2* ($^{2}Q_{26}$) and *Csn1s1* ($^{2}Q_{44}$) genes, respectively. This loss in accuracy of the splicing machinery would be due to the nucleotide sequence at the intron-exon junction. The mechanism by which the 3′ splice site AG is accurately and efficiently identified involves a 5′-to-3′ scanning process. The first AG downstream from the branch point-polypyrimidine tract is preferentially selected. A second AG, competitive with the proximal one, can be used alternatively (Smith et al., 1993). Starting the exon sequence with a CAG (coding for a glutaminyl residue) would be a facilitating situation. The short size of the intron might be an enhancing factor. Indeed, introns 6 and 10, involved in human and ruminant genes encoding α_{s1}-casein, are 150 bp (Martin et al., 1996) and 100 bp long (Koczan et al., 1991; Leroux et al., 1992), respectively. Likewise, in mice, upstream introns are 81 (*Csn2*, exon 6) and 84 bp (*Csn1s1*, exon 8) long, respectively.

"Species-Specific" Casual Exon Skipping

Structural characterisation of caseins and/or analyses of relevant mRNA have enabled the identification, in the four ruminant species, of multiples forms of α_{s1}-casein. However, the extent of this heterogeneity depends on the species. Whilst α_{s1}-casein phenotypes consist of a mixture of two forms (199 and 198 amino acid residues) in cattle and water buffalo, due to the alternative deletion of Q_{78}, there are, in sheep and goats, at least seven molecular forms which differ in their peptide chain length, regardless of genetic polymorphism (Ferranti et al., 1997). The main component corresponds to the 199-residue form initially described in goat milk (Brignon et al., 1989). The others, in lower amounts, are shorter forms of α_{s1}-casein differing in deleted sequences (residues 110–117 and/or 141–148). Genomic and mRNA analyses demonstrated that these forms originated from exon-skipping events affecting exon 13 (encoding peptide 110–117) and/or exon 16 (encoding peptide 141–148) during the processing of the primary transcripts. Deletions of peptide 110–117, which contains 4 charged residues (S^{P}_{115}, E_{110}, E_{117} and K_{114}) and of peptide 141–148, which contains only one (E_{141}), produce proteins with a different net charge. The protein lacking sequence 141–148 is the only one to date identified and localised in isoelectric focusing in sheep (Chianese et al., 1996).

[1]Exon 8 if we adopt, for comparative purposes, a generic exon numbering valid for all species for which *CSN1S1* gene sequence is known. Otherwise it is exon 7 in the mice gene.

[2]Referring to the mature protein sequence.

The alternative splicing of exon 13 and/or 16 reported for small ruminant species has not been detected in cattle and water buffalo (Ferranti et al., 1999). Such differences in the processing of α_{s1}-casein pre-messengers in these closely related species are amazing and hard to explain, since none of the mutations (substitution or deletion) identified between small ruminants and cattle affects consensus splice sites (Leroux 1992). However, according to Passey et al. (1996), a substitution within the donor splice site could be responsible for this casual skipping, estimated to affect 20% of the total ovine α_{s1}-casein mRNA, through the formation of an inhibitory RNA secondary structure. Long and short variants of α_{s1}-casein which differ by the presence or absence of a stretch of 8 amino acid residues encoded by exon 16 have been observed in camel milk also (Kappeler et al., 1998).

This phenomenon is clearly not restricted to ruminants. The existence of three α_{s1}-casein transcripts has been reported in human mammary tissue (Johnsen et al., 1995; Martin et al., 1996). This heterogeneity is due to a differential splicing of exon 7 (bovine gene numbering) and to the usage of a cryptic splice site. Likewise, porcine α_{s1}-casein also shows such heterogeneity (Alexander et al., 1992) with multiple forms differing by internal deletion. However, exons (12 and 13′, using the bovine gene numbering) showing such a casual alternative splicing are different from those reported for the other species. Exon-skipping events affecting exons 9, 10, 16.3,[3] 16.14[3] and 17 have been reported recently in mice (Boumahrou et al., 2011).

Multiple forms arising from casual alternative splicing have been reported also in ovine α_{s2}-casein (Boisnard et al., 1991). Two non-allelic forms of α_{s2}-casein differing by an internal deletion of nine amino acid residues at positions 34–42 in the peptide chain have been found in ovine milk. Analysis of the products obtained by reverse transcription of mRNAs has shown greater heterogeneity of α_{s2}-casein transcripts. In addition to the expected deletion of codons (34–42) affecting 30–40% of mRNA, another structural difference involving an internal stretch of 44 nucleotides in the 5′-UTR has been reported subsequently to be caused by casual exon skipping.

Exon skipping has therefore to be considered as a frequent event, mainly in the case of α_{s1}- and α_{s2}-casein genes, for which the coding region is divided into many short exons. However, since these pioneering works, many additional examples have been found, and the existence of intronic *cis*-element (intronic splicing enhancer) increasing the inclusion of "weak" exons or influencing cryptic splice site usage has been reported in the equine β-casein gene (Lenasi et al., 2006). Do those deletions in calcium-sensitive caseins simply reflect the lack of accuracy of an intricate processing mechanism whenever mutations induce conformational modifications of pre-mRNA, preventing or enhancing the normal progress of events? Notwithstanding, these phenomena are mainly responsible for the great complexity of casein composition.

Genetic Polymorphisms Increase Casein Heterogeneity in Peptide Chain Length

In addition to casual exon skipping, genetic polymorphism of milk proteins may sharply increase the heterogeneity of caseins in milk. Studies performed on goat milk have reported extensive genetic polymorphism of α_{s1}-casein with at least 15 alleles at the goat α_{s1}-casein (*CSN1S1*) locus, distributed in seven different classes of protein variants associated with four levels of expression (Bevilacqua et al., 2002). α_{s1}-Casein A, B, C and E variants differ from each other in amino acid substitutions, whilst α_{s1}-casein variants F and G, which are associated with a low level of protein synthesis, are internally deleted (Martin 1993; Martin and Leroux 1994). The establishment of the overall organisation of the goat α_{s1}-casein gene (19 exons scattered along 17 kb), the characterisation of allele F (Leroux et al., 1992) at the genomic level as well as the analysis of their transcription products demonstrated that the internal deletion of 37 amino acid residues, occurring in variant F, arises from the outsplicing of three consecutive exons (9, 10 and 11), skipped *en bloc* during the processing of the primary transcripts

[3]Refers to tandem repeats of exon 16, using the generic exon numbering valid for all species.

(Fig. 13.9). Furthermore, the *CSN1S1*F* allele was shown to yield multiple alternatively spliced transcripts, amongst which were transcripts lacking 24 nucleotide-long sequences encoded by exons 13 and 16 (Leroux et al., 1992). By comparison with the non-defective *CSN1S1*A*, *B* and *C* alleles, a reduction in the amount of mRNA, due to mRNA decay and therefore accounting for lower α_{s1}-casein content in milk, was observed. A single point deletion in exon 12 of the α_{s1}-casein gene, leading to truncated proteins and hence a low content of α_{s1}-casein in the milk, has been described as being unique to the Norwegian goat population (Hayes et al., 2006).

Likewise, a single-nucleotide deletion resulting in a premature stop codon is associated with a marked reduction in the amount and an extensive heterogeneity of transcripts from goat β-casein (*CSN2*) null allele in Créole and Pyrenean breeds (Persuy et al., 1996, 1999). These authors have shown the occurrence of multiple and shorter transcripts which differ from their full-length counterparts in large nucleotide stretches that were missing in exon 7. Four in-frame nonsense codons, due to a one- nucleotide deletion, were found in the *CSN2*0* allele and the cognate mRNA. Another β-casein null allele identified in a Neapolitan goat breed (Chianese et al., 1993) was shown to differ from the wild type by a transition C→T, affecting codon 157 in exon 7 (Rando et al., 1996). The resulting premature termination codon is associated with a tenfold decrease in β-casein mRNAs. However, data are lacking about the possible occurrence of multiple mRNAs.

As regards both *CSN1S1*F* (as well as the Norwegian *CSN1S1* allele) and *CSN2*0*, mutations are responsible for the existence of premature stop codons, associated with a decrease in the relevant level of transcripts and responsible for the presence of multiple forms of messengers, due to alternative splicing. Many reports (reviewed by Valentine 1998) have drawn attention to a possible relationship between nonsense codons and exon skipping. Indeed, some genes containing premature codons express alternatively spliced mRNA in which the exon containing the nonsense codon has been skipped. Amongst the hypotheses proposed to explain such a safeguard mechanism, one can mention "nuclear scanning" which recognises nonsense codons and then has an effect on exon definition (Zhang et al., 1998a, b). This raises the question: How is a normal termination codon (which does not usually mediate a reduction in the abundance of mRNA) distinguished from a premature stop codon? A rule has been proposed for the position of the termination codon. According to Nagy and Maquat (1998), it must be located less than 50–55 nucleotides upstream from the 3′-most exon-exon junction. The normal termination codon in α_{s1}-casein, as well as in β-casein transcripts, is in part or fully encoded by the penultimate exon (exons 18 and 8, respectively) at 43 and 36 nucleotides upstream from the last exon-exon junction, respectively, thus conforming perfectly with the stated rule. Conversely, stop codons identified both with *CSN1S1*F* and the French and Italian *CSN2*0* alleles are located well beyond the 55 nucleotide limit. Therefore, they could be suspected to mediate mRNA decay and promote the occurrence of multiple forms of transcripts.

13.2.3.2 Differences in Post-translational Modifications

Our present knowledge of casein heterogeneity is rather advanced for a large number of species, including cattle, goats and even for, until now, less thoroughly investigated species such as sheep, humans or horse. With the growing resolving power of 2D-electrophoretic techniques, the development of immunochemical procedures, coupled with gel electrophoresis and the increasing usage of mass spectrometry-based proteomics, we now have a clear vision of the complexity of the casein fraction in most of the species studied so far. Genetic polymorphisms remain one of the factors determining casein heterogeneity through alterations in electrical charge, molecular weight and hydrophobicity of proteins. However, other factors such as PTM, including phosphorylation and glycosylation (κ-casein), also contribute significantly. These factors will be examined below.

Phosphorylation

Phosphorylation of caseins is a post-translational event occurring in the Golgi apparatus and catalysed by specific kinase(s) that recognises an amino acid triplet where the determinants are dicarboxylic residues (mainly Glu) or phosphoseryl residues (Mercier 1981). The occurrence of the tripeptide sequences Ser-X-Glu/SerP is a necessary but not a sufficient condition for phosphorylation of caseins to occur. Possible factors of constraint such as different intrinsic properties of both phosphate acceptor residues and acidic determinants, the characteristics of the local environment, secondary structure and steric hindrance, an insufficient available pool of kinase(s) may explain incomplete phosphorylation.

Indeed, unlike milk from various ruminant species, human and equine milks display complex phosphorylation patterns (Poth et al., 2008; Matéos et al., 2009). Whilst bovine β- and α_{s1}-caseins exist either predominantly as single phosphoforms, containing 5 and 8 phosphate groups, respectively, other mammals, in contrast, have more variable phosphorylation forms. For instance, equine and human β-caseins have variable phosphorylation levels with 3–7 and 0–5 phosphates, respectively (Girardet et al., 2006; Greenberg et al., 1984). This notion, however, has to be considered cautiously since in ewe's milk, α_{s1}-casein has been reported to exist as multiple phosphoforms with 7–11 phosphate groups, (Mamone et al., 2003). This is also true for β-casein (2–7 phosphates).

Our current knowledge on the phosphorylation level of the main 4 caseins from 12 mammals is gathered in Table 13.2. The greatest amount of data refers to the four widely studied ruminant species, whilst only limited information is currently available regarding the other species, with the exception of human and horse. Experimental data are compared to the theoretical number of sites expected on the basis of Mercier's rule.

Five variants of ovine α_{s1}-casein (A to E) have been described so far, associated with quantitative variation in casein content (Chianese et al., 1996). The primary structure of three of them, A, C and D (formerly called Welsh variant), has been determined. They differ from each other by few amino acid substitutions and the degree of phosphorylation (Ferranti et al., 1995). Differences between the three genetic variants, A, C and D, are "silent" substitutions that affect the degree of protein phosphorylation: Variant C differs from variant A for the substitution Ser_{13}→Pro, which determines the loss of a phosphate group at site 12 of the peptide chain, $PSer_{12}$→Ser; a further substitution, $PSer_{68}$→Asn, causes the disappearance of the phosphate group on both phosphorylated residues, Ser_{64} and Ser_{66}, in variant D which is widespread in Italian breeds (Russo and Davoli 1983).

As for other species, ovine α_{s2}-casein appears to be the most heterogeneous fraction due to its high degree of multiphosphorylation, with 9–12 phosphate groups (Mamone et al., 2003).

In sows' milk, polymorphism of α_{s2}-casein consists of a fast-migrating band with a minor satellite band which is absent from some samples (Erhardt 1989). The author suggested that this was determined by the incomplete phosphorylation of potentially phosphorylable sites which are saturated only in the case of bovine and water buffalo α_{s0}-casein.

Camel milk caseins are less phosphorylated than bovine caseins (Kappeler et al., 1998). Six putative phosphorylation sites (Ser at positions 18, 68 and 70–73) have been identified in camel α_{s1}-casein, with a possible incomplete saturation. Although there are four predicted phosphorylation sites in the β-casein peptide sequence, from molecular mass measurements (MALDI-MS), it was observed that the most frequent form has only three phosphate groups. One deletion that shortens camel α_{s2}-casein, likely due to the skipping of exon 8, is responsible for the loss of the phosphorylated serine cluster Ser_{56}-Ser_{57}-Ser_{58}.

Two phosphorylation sites had been identified to date ($SerP_{151}$ and $SerP_{168}$) out of five potentially phosphorylable sites (also including Ser_{127}, Thr_{135} and Thr_{137}) in the bovine κ-casein peptide chain. Holland et al. (2006), using a proteomic approach (2D-electrophoresis, combined with mass spectrometry) to analyse the casein fraction of milk from a single cow, homozygous for the B variant of κ-casein, have characterised 17 isoforms with different PTM and were able to identify a previously unrecognised site (Thr_{166}) that could be phosphorylated or glycosylated.

Table 13.2 Main structural features of the caseins from 12 placental mammalian species

	Species												
Caseins	Cattle	Water buffalo	Sheep	Goat	Pig	Camel	Horse	Rat	Mouse	Guinea pig	Rabbit	Human	Features
CSN1S1	199	199	199	199	191	215	197	187	187	179	197	170	Mature protein (n)
	15	15	15	15	15	15	15	15	15	15	15	15	Signal peptide (n)
	9/9	8/8	10/10	11/11	7/?	6/?	10/?	3/?	4/?	12/?	9/?	6/0–8	Phosphorylation sites (p/a)
CSN2	209	209	207	207	217	217	226	216	214	/	222	212	Mature protein (n)
	15	15	15	15	15	15	15	15	15	/	15	15	Signal peptide (n)
	6/5	5/5	6/6	6/6	5/?	4/?	9/7	11/?	9/?	/	4/?	6/6	Phosphorylation sites (p/a)
CSN1S2	207	207	208	208	220	178	/	163	128–169	209	166	/	Mature protein (n)
	15	15	15	15	15	15	/	15	15–15	15	15	/	Signal peptide (n)
	17/?	17/?	17/13	16/?	18/?	9/?	/	14/?	6/?–16/?	20/?	7/?	/	Phosphorylation sites (p/a)
CSN3	169	169	171	171	167	162	185	159	160	213	160	162	Mature protein (n)
	21	21	21	21	21	20	21	21	21	21	21	20	Signal peptide (n)
	5/3	5/3	5/3	6/3	?/?	?/?	2S+6T/?	2/?	2/?	9/?	5/?	5/?	Phosphorylation sites (p/a)

For each species, the number of amino acid residues of the mature chain and of the signal peptide, as well as the number of phosphorylation sites (putative/effective), are indicated

Glycosylation

Ovine κ-casein is *O*-glycosylated, and Thr residues at positions 156, 158 and 159 have been proposed as putative glycosylation sites (Fiat and Jolles 1989). The oligosaccharide units of ovine κ-casein contain both *N*-acetyl and *N*-glycolyl neuraminic acids at all stages of lactation (Jollès and Fiat 1979; Soulier et al., 1980; Soulier and Gaye 1981). The disaccharide, Gal β (1→3) GalNAc, and the tetrasaccharide, Gal β (1→3) [Gal β (1→4) GlcNAc β (1→6) GalNAc], occur in mature ovine κ-casein, whereas defined tetra- and penta-saccharide structures are present in κ-casein from colostrum, indicating an evolution of the sugar moiety as a function of the time after parturition. In a comparative study, the caseinoglycopeptide from ewes' milk was shown to have greater antithrombotic activity than that of the cow (Bal dit Solier et al., 1996).

The carbohydrate content of κ-caseinoglycopeptide is significantly higher in human (55%) than for bovine (*ca.* 10%). The monosaccharides, Gal, GalNAc and NeuAc, are common to the κ-casein from both species, whereas Fuc and GlcNAc are specific of human κ-casein (van Halbeek et al., 1985). An increasing number of oligosaccharide structures for human κ-caseinoglycopeptide is available (Saito et al., 1988; Saito and Itoh 1992). Amongst these oligosaccharides, GlcNAc β (1→6) GalNac and GalNAc β (1→4) GlcNAc β (1→6) GalNAc represent novel types of core structures for mucin-type carbohydrate chains (Fiat and Jolles 1989).

Although the primary structure of κ-casein from several species is available, glycosylation sites are essentially documented for bovine and human κ-caseins (Pisano et al., 1994). Ser residues were not glycosylated in bovine or human κ-casein (Fiat et al., 1980), whereas nine out of ten putative Thr-containing consensus sequences of human κ-casein are actually glycosylated. Potentially, bovine κ-casein could have up to 12 NeuAc residues if all six glycosylation sites (only five in the B variant) were modified with the major tetrasaccharide NeuAca(2–3)Galβ(1–3) [NeuAca(2–6)]GalNAc, identified in κ-casein. However, using 2D-electrophoresis coupled with mass spectrometry, only fragment masses corresponding to a maximum of four NeuAc residues were observed in the multiply glycosylated forms (Holland et al., 2004a, b). On the other hand, the core disaccharide, Galβ(1–3)GalNAc, to which the NeuAc residues are attached, appeared to be relatively stable, and fragment ions with up to three disaccharides were observed allowing the number of oligosaccharides attached to be determined.

13.2.4 Impact on Micelle Organisation

Although it is not within the scope of this chapter, one cannot ignore the effect of casein structure and diversity on the characteristics and behaviour of the casein micelle. Interspecies comparison, as well as genetic polymorphisms (Martin et al., 1999), must be considered as a valuable tool for probing the overall organisation of the casein micelle and to extending our understanding of the mechanisms involved in its formation. Relative proportions of caseins, which is a specific trait, their intrinsic characteristics (Table 13.2), essentially determined by their primary structure, are amongst the many factors that will determine, in each species, the average size (diameter and size distribution) of the casein micelle, its surface charge and hydrodynamic radius, its hydration and mineral content.

Several experiments (Heth and Swaisgood 1982; Donnelly et al., 1984; Dalgleish et al., 1989) have led to the conclusion that the average size of the micelle increases as the proportion of κ-casein decreases. This finding is confirmed through interspecies comparisons, using the freeze-fracture technique (Buchheim et al., 1989). Camel and human milks, with a low (3.5%, Kappeler et al., 1998) and a high (17%, Dev et al., 1994; Miranda et al., 2000) content in κ-casein, respectively, display larger (up to 600 nm, with an average around 350 nm, as for the llama) and smaller (64 nm) casein micelles, although the higher mineral content of camel milk might also play a significant role in this regard. It seems that the casein micelles in milk from different species have a similar ultrastructure but with considerable differences in the size distribution. Bloomfield (1979) provided the

most complete theory for the origin of a broad size distribution of micelles using the model of Slattery and Evard (1973), based on the variations in subunit composition. It is clear that the occurrence of multiple molecular species of each casein, differing in their length, their sequence and their level of phosphorylation (e.g. human casein micelle; Dev et al., 1994), further complicated by genetic polymorphisms more or less pronounced within each species (e.g. the goat), is not without any consequence, as far as this issue is concerned.

In contrast, the relationship between micellar size and the proportions of the "calcium-sensitive" caseins is less clearly established. Few contradictory data are available for bovine casein micelles. For example, Davies and Law (1983) found that α_{s1}-casein is present in about the same proportion in micelle fractions isolated by differential centrifugation, whereas α_{s2}-casein increased slightly with ease of sedimentation. Conversely, Donnelly et al. (1984), using a chromatographic approach, reported that both α_s-caseins are present in greater proportions in the larger micelles. Once again, goats, with the complex (including quantitative) genetic polymorphism described at several casein loci (namely, α_{s1}, β and κ) provides a valuable tool to address this issue. Indeed, deficiency of α_{s1}-casein, together with changes in the primary structure, has been shown to be responsible for the variability in micellar diameter (Remeuf 1993; Grosclaude et al., 1994; Pierre et al., 1995). One should therefore no longer address "the" micelle structure as a singular issue but rather consider that the broad size distribution generally observed might reflect the extensive diversity of molecular species arising from the expression of each of the four (or five) casein genes.

13.3 Whey Proteins

There are a significant number of proteins in the whey, synthesised in the mammary tissue or not for which the functions are not fully understood. We will discuss briefly below variation across species, taking into account quantitative as well as structural and biological aspects of selected major whey proteins.

Variation in the milk protein gene copy number potentially contributes to the diversity of milk protein composition (Lemay et al., 2009). This is particularly well exemplified by the gene encoding β-lactoglobulin (*BLG*) which is one of the major whey protein of ruminant species, apparently absent in human, camel, rabbit and rodents (Sawyer 2003). Surprisingly, another major whey protein—the whey acidic protein or WAP—is frequently found instead BLG. However, the presence of WAP in human milk has still to be demonstrated. On the other hand, both proteins are found in marsupial and monotreme milks (Fig. 13.8). Up to now, swine is the only eutherian species for which WAP has been identified together with BLG (Simpson et al., 1998).

13.3.1 β-Lactoglobulin: A Singular and Enigmatic Whey Protein

BLG has been studied extensively across species and a comprehensive review was published few years ago (Sawyer 2003; see also Chap. 7). Despite relatively weak sequence homologies, multiple alignments reveal some striking features and short peptide sequences precisely conserved across species, including marsupials (Fig. 13.9). The genomic organisation of the gene encoding BLG is highly conserved across species, with seven exons, encompassing a *ca*. 5 kb genomic segment, located on chromosome 11 in cattle. With the release and the assembly of the *Bos taurus* genome, it appeared that the gene encoding BLG is duplicated in cattle as it is in dog and horse genomes. The duplicated gene, first described in cattle as a pseudogene (Passey and MacKinlay 1995), shows similarities to *BLG-II* genes identified in the horse and cat (Lear et al., 1999; Pena et al., 1999). However, there is no evidence for its expression in the bovine mammary gland (Lemay et al., 2009), thus being without any effect on the concentration of BLG in bovine milk. On the other hand, mutations within *BLG-I*

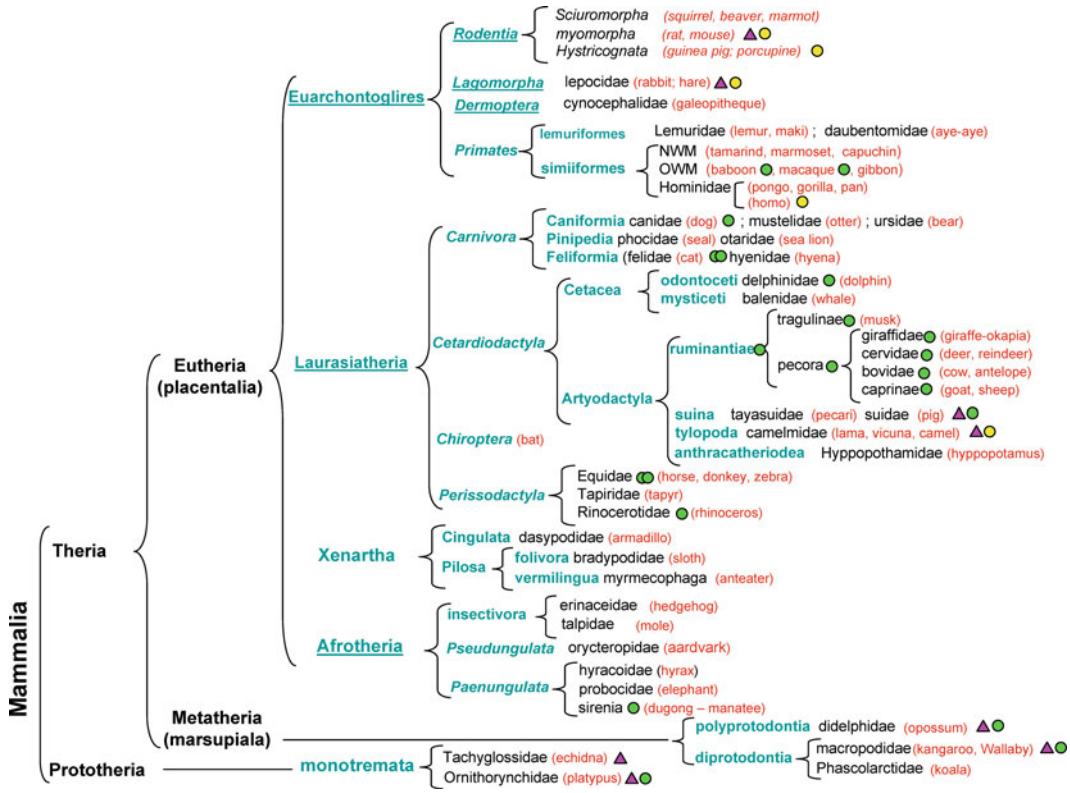


Fig. 13.8 Mammalian phylogeny and the presence of WAP/BLG in milk. Genera are given in red. NWM corresponds to New World Monkeys, whereas OWM are related to Old World Monkeys. ▲: indicates the presence of WAP in milk. ●: marks the presence of β-lactoglobulin, while ●●: indicates the occurrence of several copies of the gene encoding β-lactoglobulin in the genome. ●: marks the absence of β-lactoglobulin

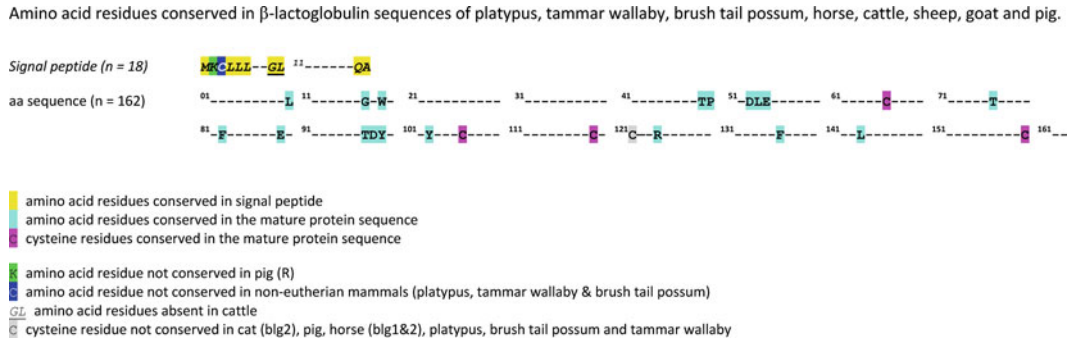


Fig. 13.9 Amino acid residues conserved in β-lactoglobulin sequences of platypus, tammar wallaby, brush tail possum, horse, cattle, sheep, goat and pig

gene, including its promoter region, seem to impact BLG expression level.

The amino acid sequence and 3-dimensional structure of BLG show that this protein belongs to the widely diverse lipocalin superfamily, the members of which share relatively low sequence similarity but have a highly conserved exon/intron structure and three-dimensional protein folding. Most of them bind small hydrophobic ligands and thus may act as specific transporters, as does

serum retinol binding protein. Bovine BLG binds a wide range of ligands, but this may not be the reason for its presence in milk. The structure and physicochemical properties of the protein have been reviewed by Kontopidis et al. (2004). The apparent ability of the binding site to accommodate a wide range of ligands may point to a possible physiological function. However, by considering the lipocalin family, in general, and the species distribution of BLG in particular, some speculation can be made. It has been reported as being implicated in hydrophobic ligand transport and uptake, enzyme regulation and the neonatal acquisition of passive immunity. However, these functions do not appear to be consistent between species. Sequence comparisons amongst members of the lipocalin family reveal that glycodelin (also known as PP14, placental protein 14 or PAEP, progestogen-associated endometrial protein), found in the human endometrium during early pregnancy, is the most closely related to BLG. Although the function of glycodelin is not fully elucidated, it appears to have essential roles in regulating a uterine environment suitable for pregnancy and possibly to have effects on the immune system and/or to be involved in differentiation (Kontopidis et al., 2004).

Several polymorphic variants of BLG are known in cattle (Farrell et al., 2004; Table 13.1), but the most frequent two (A and B) were shown to be associated with differences in milk protein yield and composition. These variants differ by two amino acid substitutions in the polypeptide chain arising from two single-nucleotide substitutions in *BLG-I* gene: Asp 64 (G<u>A</u>T)→Gly (GGT) and Val 118 (G<u>T</u>C)→Ala (GCC). The latter T→C transition creates a *Hae*III restriction site, thus enabling a restriction fragment length polymorphism analysis at the *BLG* locus (Medrano and Aquilar-Cordova 1990).

Quantitative effects of these common variants on milk composition and cheesemaking properties have been reported (Aleandri et al., 1990). Allele B of *BLG* is associated with high casein and fat contents in cows' milk, whilst Holstein cows with AA genotype at the *BLG* locus were shown to produce milk containing more whey and total proteins than those of the other genotypes. This question and the impact of milk protein variants, including BLG, on milk composition have been studied extensively (for a review, see Martin et al., 2002 and Chap. 15). Two studies dealing with this issue in cattle have been published recently (Heck et al., 2009; Hallén et al., 2008). In addition, a higher expression of allele A has been described in heterozygous (AB) animals (Graml et al., 1989). This differential allelic expression has been explained by nucleotide differences in the promoter regions associated with these two alleles. Wagner et al. (1994) identified 14 single-nucleotide polymorphisms (SNP) within the 5′-flanking region and two in the 5′-UTR of exon 1 of the bovine *BLG* gene. Some of them are located in potential binding sites for trans-acting factors or in the 5′-UTR. Sequences of the 5′-flanking regions and *BLG* genotypes suggest that alleles *A* or *B* in the coding regions were connected with distinct promoter variants. Such intragenic haplotype associations may explain the observed differences in the effects of A or B variants of BLG on milk production traits particularly on BLG synthesis (A>B) in heterozygous cows (Graml et al., 1989).

By sequence analysis of the 5′-flanking regions of the milk protein-encoding genes altogether 65 variable sites have been revealed by Geldermann et al. (1996). Sixty of these sites were base substitutions, and five were deletions/insertions. About 50% of the variable sites were located in potential protein binding sites, identified by computer-aided analysis. In cell culture tests, the investigated promoter variants led to different reporter gene expression. In the case of the BLG encoding gene, the promoter variant of the *BLG***A* allele produced up to 3.5 times greater expression of a reporter gene than the promoter associated with the *BLG***B* allele. Folch et al. (1999) also showed differential expression of a reporter gene fused to bovine *BLG***A* or *B* promoters in transiently transfected HC11 cells; the A promoter driving more efficient expression of the reporter than the B (57% vs. 43%).

More recently, Braunschweig and Leeb (2006) have shown the existence of a C to A transversion at position 215 bp upstream the translation initiation site (g.-215C>A), segregating perfectly with a differential phenotypic expression of two

*BLG*B* alleles (B and B*). The sequence of the *BLG*B* allele in the region of the mutation is highly conserved amongst four related ruminant species. The mutation site corresponds to a putative consensus-binding sequence for transcription factors c-Rel and Elk-1. These results support the hypothesis according to which sequence variation within the promoter of the *BLG* gene is probably one of the factors responsible for differences in BLG content in milk.

13.3.2 Whey Acidic Protein (WAP)

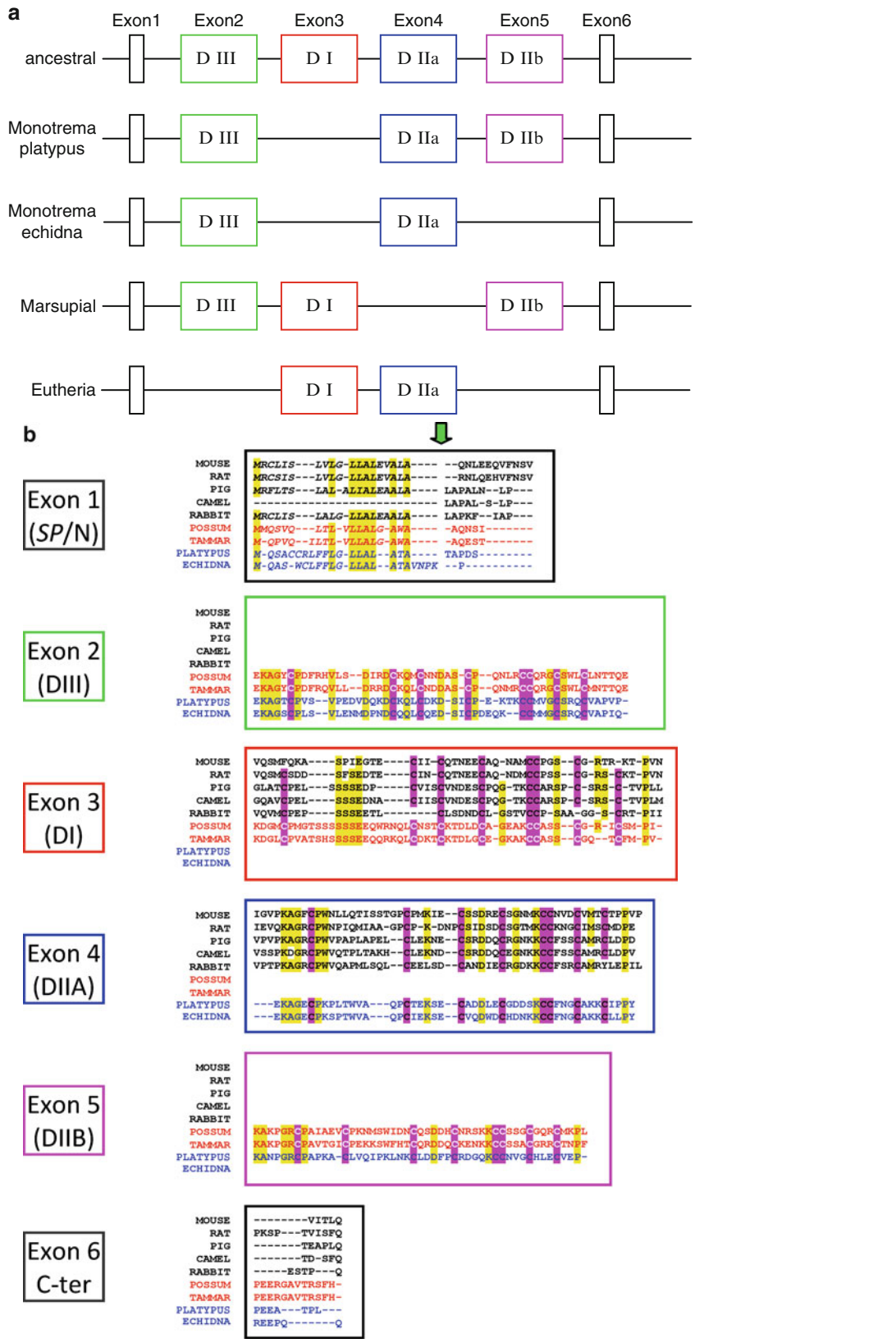
Whey acidic protein has been identified in the milk of only a few mammalian species, including mouse, rat, rabbit, camel, pig, tammar wallaby, brushtail possum, echidna and platypus, but it is absent from ruminant milks due to a frameshift mutation in the WAP encoding gene (Hajjoubi et al., 2006). The three ruminant WAP sequences have the same deletion of a single nucleotide at the end of the first exon when compared with the pig sequence. Due to the induced frameshift, the putative proteins encoded by these sequences do not harbour the features of a usual WAP protein with two four-disulfide core (4-DSC) domains, approximately 50 amino acids which contain eight cysteine residues in a conserved arrangement (Hennighausen and Sippel 1982; Ranganathan et al., 2000). Moreover, RT-PCR experiments have shown that these sequences are not transcribed. This loss of functionality of the gene in ruminants raises the question of the biological role of the WAP.

The WAP proteins share limited amino acid sequence identities with the exception of these cysteine residues (at least one, usually two 4-DSC domains in eutherian and even three in metatherian mammals) and positional conservation of several proline (P), glutamic acid (G), aspartic acid (D) and lysine (K) residues (Simpson and Nicholas 2002). Unlike the eutherian WAP sequences, marsupial WAPs display a conserved motif (KXGXCP) at the beginning of each 4-DSC domain (Fig. 13.10). However, currently no functional significance has been ascribed for this motif, although it is proposed to be important for correct folding of the protein (Ranganathan et al., 2000). The presence of 4-DSC domain sequences on chromosome 20 (WFDC2 or HE4 protein) within the human genome, raises the possibility (not yet demonstrated) that a secreted WAP protein may be present in human milk.

WFDC2/HE4 protein is a small secretory protein shown to function as an anti-proteinase (protease inhibitor) involved in the innate immune defence of multiple epithelia (Bingle et al., 2006). The relevant gene is highly expressed in pulmonary epithelial cells, in saliva and was also found to be expressed in some ovarian cancers and epididymis.

The organisation of eutherian *WAP* genes is highly conserved and composed of four exons with exon 1 encoding the 5′-UTR, signal peptide and first 8–10 amino acids of the mature protein. Exons 2 and 3 encode the two 4-DSC domains,

Fig. 13.10 Schematic representation of the structure and evolution of the whey acidic protein (WAP) gene (adapted from Sharp et al., 2007). (**a**) The ancestral progenitor is depicted by six exons (*boxes*) numbered from 1 to 6. *Coloured boxes* represent exons (2–5) encoding 4-DSC domains, *whilst black boxes* represent exons encoding the signal peptide (*SP*) and the N-terminal part (N) of the mature protein (exon 1) and the C-terminal part (C-ter) of the protein (exon 6). In eutherian WAP, 4-DSC domains (DI and DIIa) are encoded by exons 3 and 4. The two 4-DSC domains of echidna WAP are encoded by exons 2 (DIII) and 4 (DIIa). Marsupial and platypus WAPs comprise three 4-DSC domains in different configurations: DIII—DI—DIIb and DIII—DIIa—DIIb, respectively. Evolution of *WAP* genes in mammalian species would have proceeded step by step by loss of exon to lead to the present *WAP* genes in monotremes (platypus and echidna), marsupial and placental mammals. (**b**) Alignment of eutherian, marsupial and monotreme WAP sequences shows conservation of protein structure. The 4-DSC domains represented by 8 cysteine residues (C) are highlighted with a *pink background* in each domain (exon). Highly conserved residues are highlighted with a *yellow background*. Mouse (P01173), rat (P01174), pig (O46655), camel (P09837) and rabbit (P09412) for eutherian sequences have been aligned with brush-tailed possum (Q95JH3), tammar wallaby (Q9N0L8), platypus (A7J9L3) and echidna (A7J9L2) to maximise similarity within exons depicted by *boxes* for which the colour code of (A) has been retained


a
Exon1
Exon2
Exon3
Exon4
Exon5
Exon6
ancestral
D III
D I
D IIa
D IIb
Monotrema platypus
Monotrema echidna
Marsupial
Eutheria
b
Exon 1 (SP/N)
Exon 2 (DIII)
Exon 3 (DI)
Exon 4 (DIIA)
Exon 5 (DIIB)
Exon 6 C-ter
MOUSE
RAT
PIG
CAMEL
RABBIT
POSSUM
TAMMAR
PLATYPUS
ECHIDNA

and exon 4 encodes the last 8–10 amino acids of the protein and the 3′-UTR. Whilst the size of each exon remains rather conserved between species, intron size varies considerably. Exon 3, encoding 4-DSC domain II, has the higher degree of sequence conservation between species. It was proposed to be the primordial domain, with domain I likely to have arisen by intragenic duplication (Simpson and Nicholas 2002).

A third 4-DSC domain encoded by an additional exon has been identified in marsupial WAP, as well as in platypus (Sharp et al., 2007; Topcic et al., 2009), whereas the WAP gene structure of echidna is different and closer to that of the *WFDC2* gene, with only two 4-DSC domains (Fig. 13.10). It is possible that domain III of the marsupial WAP gene may be the ancestral gene, which was subsequently lost during evolution in eutherian species. Sharp et al. (2007) suggest that the evolution of the WAP gene in the mammalian lineage may be either through exon loss from an ancient ancestor or by rapid evolution via the process of exon shuffling.

Whereas eutherian WAP is expressed in the mammary gland throughout lactation, marsupial WAP is expressed only during mid-late lactation. This transient expression pattern in marsupials has to be correlated with a short gestation giving birth to an immature young followed by a long lactation during which milk progressively changes in composition to suit developing young requirements. This suggests that WAP may play a role in the development of the mammary gland or influence development of the young (Sharp et al., 2007). Interestingly, tammar mammary gland was shown to express strongly a second WAP-like protein (WFDC2) during pregnancy, at a reduced level in early lactation before it disappears in mid-late lactation. These different temporal expression patterns of WAP and WFDC2 suggest they play complementary roles.

13.3.3 α-Lactalbumin

Amongst the main milk proteins, α-lactalbumin (LALBA) is so far the only one with enzyme-related activity. Together with β-1,4-galactosyltransferase, it forms the lactose synthase complex which catalyses the formation of lactose from glucose and UDP-galactose, (Brew and Hill 1975) in the Golgi apparatus of MEC. α-Lactalbumin was shown to be a calcium metalloprotein, in which the calcium ion has an unusual role in folding and structure (Hiraoka et al., 1980).

Most of the molecular structure and function was known and extensively reviewed by Brew (2003; see also Chap. 8), but this protein has experienced a renewal of interest with "HAMLET" (human alpha-lactalbumin made lethal to tumour cells), a partially unfolded α-lactalbumin, which acquires, when it binds oleic acid, a tumoricidal function (Pettersson-Kastberg et al., 2009).

α-Lactalbumin is present in the milk of almost all species of mammals, except some Otariidae (*Arctocephalus pusillus*: Cape fur seal), the milk of which is rich in fat (more than 20% long-chain fatty acids, mainly unsaturated) and devoid of lactose and α-lactalbumin (Dosako et al., 1983).

The female fur seal modulates its lactation by turning milk production "on" and "off" without regression and involution of the mammary gland (Sharp et al., 2006). After undergoing a perinatal fast of 2–3 days suckling pups on shore, the mother leaves her young and the colony to forage at sea for 3 weeks to replenish body stores during which time her mammary gland remains active without initiating involution, demonstrating an apoptotic function for α-lactalbumin (Sharp et al., 2008). This apoptotic potential which is consistent with observations made on α-lactalbumin-deficient mice (Stinnakre et al., 1994) has to be compared with the tumoricidal function of HAMLET.

The gene structure and the protein sequence are highly conserved across species, with more divergence in rodents than in primates (Brew 2003).

Due to its prominent role in milk synthesis, α-lactalbumin is considered to be a valuable genetic marker for milk production traits in cattle. However, α-lactalbumin appears rather weakly polymorphic in cattle (3 variants) and sheep (2 variants). After screening at the protein, as well as the nucleotide level, few mutations have been found, mainly within the regulatory sequences of the gene.

Bleck and Bremel (1993b) sequenced the 5′-flanking region of the α-lactalbumin gene in cattle. Three SNPs occurring at positions +15, +21 and +54 relative to the mRNA transcription start point were identified within a *ca*. 2-kb fragment including 1,952 bp of 5′-flanking region and 66 bp of the protein-coding region. The +15 and +21 variations occurred in the 5′-UTR of the mRNA, whereas the +54 polymorphism is a silent mutation in the signal peptide-coding region of the gene. A transition A→G at position +15 was shown to occur only in the Holstein breed (Bleck and Bremel 1993a) and to be associated with an increased milk yield. Cows with the *A* allele of the *LALBA* gene had higher milk yield, protein yield and fat yield; the *B* allele was associated with higher percentage of protein and fat. These data suggest that although not located in the gene promoter, this SNP potentially alters α-lactalbumin expression at the translational level and may be associated with differences in milk yield.

In addition to SNP/+15, a second SNP (also a transition A→G), located at position −1,689 from the transcription start point was identified (Voelker et al., 1997). The allele showing an A at position −1,689 was designated as allele *A*, and that with a G at this position was designated allele *B*. The −1,689 and +15 polymorphisms were compared within the Holstein population to determine their linkage relationship. In this study, the +15 A variant was always linked to variant A at −1,689. These results suggest the existence of a haplotype A (+15A and −1,689A) associated with higher milk, protein and fat yields.

13.3.4 Lysozyme

Lysozyme is a bacteriocidal enzyme, structurally related to α-lactalbumin, sharing 40% similarity (Qasba and Kumar 1997). Ranging in molecular mass between 14 and 18 kDa, this enzyme, also called β-1,4-*N*-acetylmuramidase, cleaves a glycosidic linkage in the peptidoglycan component of bacterial cell walls, resulting in a loss of cell wall integrity and cell lysis. The concentration of lysozyme in milk varies from 1 to 3 mg/L in bovine milk to 400 (in human) and even 800 mg/L in mares' milk (Farkye 2003; Miranda et al., 2004).

13.3.5 Lactoferrin

Lactoferrin (LTF) is of mammary origin and is found in the milk of most species (Schanbacher et al., 1993; see also Chap. 10). LTF is an iron-binding glycoprotein with a molecular mass around 80 hDa, belonging to the transferrin family that is expressed and secreted by epithelial cells and found in the secondary granules of neutrophils from which it is released in infected tissues and blood during the inflammatory process. Initially described as an iron-binding molecule with bacteriostatic properties, LTF is now known to be a multifunctional or multitasking protein with multiple biological activities (Ward et al., 2002; Vogel et al., 2002). It is a major component of the innate immune system of mammals. Its protective effects range from direct antimicrobial activities against a large range of microorganisms including bacteria, viruses, fungi and parasites, to anti-inflammatory and anti-cancer activities. Whilst iron chelation is central to some of the biological functions of LTF, other activities involve interactions of LTF with molecular and cellular components of both hosts and pathogens (Legrand et al., 2008).

The internal structure of LTF is highly conserved and is dedicated to binding iron. On the other hand, the external structure (its molecular surface) is much more variable across species, making it more difficult to identify functionally important sites. Recent work shows that the cationic N terminus and associated lactoferricin domain on the N-lobe of LTF, in addition to its role in antibacterial activity and probable role in DNA binding, is also involved in complex formation with other proteins. Finally, it may be time to re-examine the importance of glycosylation, given the growing evidence that many pathogens depend on binding to glycans for pathogenesis (Baker and Baker 2009).

The overall structural organisation of the human, mouse, cattle, dog and horse LTF genes

is rather well conserved, at least in terms of size and number of exons ($n=17$). Indeed, LTF is encoded by an approximately 30-kb gene (ranging in size between 23.5 kb in mice and 33.4 kb in cattle), located on chromosome 3 in human, 9 in mice and 22 in cattle (Le Provost et al., 1994).

A total of 60 LTF nucleotide sequences with the complete coding regions (CDS) and corresponding amino acids belonging to 11 species were analysed recently and differences within and across species studied (Kang et al., 2008). The length of the LTF cDNA with the complete CDS varies greatly, from 2,055 to 2,190 bp, due to deletion, insertion and stop codon mutation, resulting in elongation. Observed genetic diversity was higher across species than within species, and *Sus scrofa* had more polymorphisms than any other species. Novel amino acid variation sites were detected within several species (8 in *Homo sapiens*, 6 in *Mus musculus*, 6 in *Capra hircus*, 10 in *Bos taurus* and 20 in *Sus scrofa*), illustrating functional variation.

13.4 Milk Fat Globule Membranes Proteins

Fat is present in milk as droplets of apolar lipids surrounded by a complex membrane derived from the MEC and is called MFGM. MFGM has a complex tripartite structure comprising a monolayer membrane derived from the endoplasmic reticulum (ER) surrounded by a bilayer membrane arising from the plasma membrane of the MEC. Hence, the composition of MFGM reflects those of endoplasmic reticulum and plasma membranes. Using high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS) applied to one-dimensional SDS-PAGE fractionated samples, Reinhardt and Lippolis (2006) identified more than 120 proteins in bovine MFGM with diverse functions such as trafficking, signalling or immune response. Although MFGM proteins represent only 1% of total milk proteins by weight, they possess essential roles in nutritional or technological properties of MFGM (Dewettinck et al., 2008). A great variability is observed in protein abundance. For instance, butyrophilin (BTN) accounts for up to 40% of the total protein content in the bovine MFGM. Roughly, MFGM material can be resolved by SDS-PAGE into eight protein bands corresponding to MUC-1, fatty acid synthase (FAS), xanthine oxidoreductase (XOR), MUC-15/PAS III, CD36, BTN, lactadherin (LDH) and adipophilin (ADRP). Major MFGM proteins have been reviewed extensively (Mather 2000; Keenan and Mather 2006). We will therefore focus on MFGM proteins for which recent advances have been made with special attention paid to structural and functional differences across species.

13.4.1 Mucins

Mucins are large proteins containing more than 50% *O*-glycans by weight which are present at the interface between epithelia and their extracellular environment. The extracellular part of the protein contains a domain of a tandemly repeated 20-amino acids motif known as PTS regions, which are proline, threonine and/or serine-enriched regions containing numerous *O*-glycosylation sites. Due to the anti-adhesive properties of *O*-glycans, mucins are involved in protection against infections, either caused by viral or bacterial agents (Schroten 1998; Patton 1999; Dewettinck et al., 2008). The MUC family contains more than 20 members. To date, only three mucins have been more or less extensively characterised in milk: MUC-X, MUC-1 and MUC-15 (formerly known as PASIII). MUC-X is a poorly characterised high molecular mass mucin which has been reported to be homologous to MUC-4. The presence of MUC-4 has been confirmed in human milk (Patton 2001; Zhang et al., 2005). A gene predictively encoding a mucin 4-like protein, highly homologous to the human, dog and mouse counterpart has been found on chromosome 1 in the *Bos taurus* genome (NCBI, GeneID: 786701). However, efforts need to be made to characterise MUC-4 in milk through species at the protein level. We will therefore focus on two well-described mucins in milk: MUC-1 and, more recently, MUC-15.

13.4.1.1 MUC-1

MUC-1 is undoubtedly the best characterised milk mucin. Bovine MUC-1 is a protein of 580 amino acids residues including a signal peptide of 22 residues. General features of the protein are a large extracellular region (467 amino acids) with partially conserved tandem repeats (20 amino acids each), a membrane-proximal SEA module which is a 120-amino acid domain frequently associated with heavily *O*-glycosylated proteins, a transmembrane region and a short (70 amino acids) cytoplasmic tail (Pallesen et al., 2001). The primary sequence of bovine MUC-1 has a relatively low level of homology to those for human and mouse MUC-1, with similarities of 52% and 46%, respectively. However, these values increase to 77% and 79% when amino acid sequence of the cytoplasmic tail of bovine MUC-1 is compared to its human and murine counterparts, thus suggesting a key function for this region. Indeed, the cytoplasmic part of MUC-1 has been shown to be involved in numerous intracellular signalling pathways (Singh and Hollingsworth 2006).

Because each codominant allele may contain a variable number of repeats encoding the 20-amino acids motif, different sizes of MUC-1 are observed by SDS-PAGE. Heterozygous individuals display two bands for MUC-1 on SDS-PAGE, whereas a single band is observed for homozygous individuals. MUC-1 polymorphism has been evidenced for human, chimpanzee, horse, cat and dog mucins (Spicer et al., 1991). In contrast, the polymorphic nature of the gene has been lost in the mouse and other rodents. The number of tandem repeats truly represents a matter of interspecies differences. In humans, the number of tandem repeats varies from 21 to 125 with 41 and 85 repeats being the most frequent motif encountered in the Northern European population (Gendler et al., 1990). As a consequence, the apparent molecular mass in SDS-PAGE for human MUC-1 range between 240 and 450 kDa, whereas that for bovine MUC-1 seems to be considerably lower (Pallesen et al., 2001). PCR analysis of genomic DNA from 630 individuals identified nine allelic variants spanning 7–23 VNTR units, each encoding 20 amino acids, in Holstein-Friesian cattle (Sando et al., 2009). Three alleles, containing 11, 14 and 16 VNTR units, respectively, were predominant. In addition, a polymorphism in one of the VNTR units has the potential to introduce a unique site for *N*-linked glycosylation. MUC-1 appears highly glycosylated, primarily with *O*-linked sialylated *T*-antigen [Neu5Ac(α2-3)-Gal(β1-3)-GalNAcα1] and, to a lesser extent, with *N*-linked oligosaccharides, which together account for approximately 60% of the apparent mass of the protein (Sando et al., 2009).

We recently confirmed the polymorphic aspect of MUC-1 in goat milk (presence of one or two equally PAS stained bands for MUC-1) previously demonstrated by Campana et al. (1992). In addition, we also confirmed that in comparison to its bovine counterpart, goat mucin is a considerably larger protein (Cebo et al., 2009).

Polymorphism was shown to be variable amongst species. In the goat, where high homology was observed between VNTR repeats, 15 alleles were identified (Sacchi et al., 2004). In contrast, in the ovine species where average homology between repeats was lower, only four alleles could be identified. Thus, the conservation between repeats seems to be positively correlated with the degree of polymorphism observed (Rasero et al., 2007). Additional evidence for the relationship between homology of repeats and degree of polymorphism exists in mice. Indeed, murine Muc-1, which displays only 75% homology in the repetitive domain, is not polymorphic (Spicer et al., 1991).

13.4.1.2 MUC-15

Separation of MFGM proteins by SDS-PAGE followed by PAS staining reveals the existence of another heavily glycosylated protein, MUC-15, previously known as PASIII in bovine MFGM (Mather 2000; Keenan and Mather 2006). Although the extracellular part of the protein lacks the typical tandem repeats which are hallmarks of mucins, MUC-15 contains regions rich in proline, threonine and serine residues with several potential glycosylation sites and therefore

belongs to the mucin family (Pallesen et al., 2002). Recently, the same authors confirmed the presence of MUC-15 orthologs in ewe and goat milks by purification and N-terminal sequencing (Pallesen et al., 2008). By western blotting using antibodies raised against a 15-amino acids region conserved in human, mouse and bovine MUC-15, a 130 kDa band was observed for bovine, caprine, ovine, porcine and buffalo milks, whereas a higher molecular mass (150 kDa) band was observed for human milk (Pallesen et al., 2008). Because the calculated molecular weight deduced from primary sequence is 36,294 Da for human MUC-15 and 35,715 Da for its bovine counterpart, it is likely that discrepancies in SDS-PAGE mobilities observed for MUC-15 proteins are due to different glycosylation patterns. Accordingly, alignment of amino acids sequences of human, bovine, mouse, rat and chimpanzee MUC-15 showed between 55 and 98% similarity (Fig. 13.11). The region showing the lowest conservation between species corresponds to the extracellular part of the protein. The cytoplasmic tail of MUC-15 is more conserved, thus suggesting the existence of a functional domain as a common feature. Indeed, structural motifs linking MUC-15 to the Ras intracellular signalling pathway were identified as previously shown for MUC-1 (Singh and Hollingsworth, 2006; Pallesen et al., 2008).

13.4.2 Non-mucin Proteins

13.4.2.1 Butyrophilin

BTN belongs to the B7/BTN-like proteins, a subset of the immunoglobulin superfamily. The main features of BTN are an extracellular part containing two Ig-like domains, a short transmembrane region and a long carboxy-terminal cytoplasmic domain called B30.2 domain. Interestingly, the *BTN* genes cluster is located close to the leukocyte antigen class I genes on human chromosome 6, thus linking BTN to other proteins involved in the immune response (Rhodes et al., 2001). The sequence of bovine BTN displays 71%, 84% and 97% homology with mouse, human and goat sequences, respectively. However, sequence homologies of the B30.2 domain are considerably higher across species, thus suggesting a conserved functional role for this region. BTN has been shown to be essential for the regulation of milk lipid droplet secretion, since lactation was severely compromised in mice with an ablated *Btn1a1* gene (Ogg et al., 2006). Two models are currently proposed for milk fat secretion. The prevailing model favours that a supramolecular complex between BTN, XOR and adipophilin at the surface of lipid droplet may initiate the budding of lipid droplet at the apical plasma membrane and their release as fat globules into milk (Mather and Keenan 1998). This model is currently challenged by several studies suggesting that BTN homophilic interactions solely orchestrate fat globule extrusion from mammary cells (Robenek et al., 2006). However, direct evidence of binding of BTN to XOR through the conserved B30.2 domain has been recently reported (Jeong et al., 2009). Arguing for the existence of a high degree of sequence homology between B30.2 domains from different species, the authors demonstrated that the binding was species independent, since xanthine oxidase from mice binds to B30.2 domain of bovine or human BTN (Jeong et al., 2009). Recently, the existence of polymorphism in the BTN gene was suggested (Bhattacharya et al., 2007). Comparisons of DNA sequences of exon 8 from sheep, cow and buffalo BTN gene revealed the existence of two alleles A and B, and three corresponding genotypes (AA, BB and AB) in the considered species. Interestingly, these authors suggested a relationship between genotypes and levels of milk fat secretion and/or size of fat globules through species. However, some differences exist at the molecular level for BTN, across species (Fig. 13.12). We have recently shown that BTN from bovine and caprine milks displays different apparent mobilities in SDS-PAGE after PAS staining or immunoblotting with specific antibodies (Cebo et al., 2009). Since the molecular weight deduced from primary sequences of bovine (accession number P18892) or caprine (accession number A3EY52) BTN are quite similar (59 kDa), we hypothesised that different apparent molecular weights in SDS-PAGE

```
mouse           MLTLAKIALISSLFISLPFARPQKQNPRRNVTQHTIEDVKIMRNNSIHLERSINVTSENG 60
rat             MLTLAKIALISSLFISLPFGRPQKQNLRRNVTQHTIEDVKTMRNKSIHLERRINATSENR 60
cow             MLTSAKILLISILSSLLLFGSHGEEGQKTNTTESTAEDLKTMENQSVPLESKANLTSDKE 60
human           MLALAKILLISTLFYSLLSGSHGKENQDINTTQNIAEVFKTMENKPISLESEANLNSDKE 60
                **: *** *** *   *  .   ::.   *.*:   * .* *.*:.: **   * .*::


mouse           S-DISNLMVTTPSPLNLS-TTFRTTNSTRTWLMTSSSESSRPSSTYS-VPPLVQGFVSKL 117
rat             S-DISNLMVTAPSLLDLS-TTFKATNSSRNFPTASSTESPKPPSTHS-IPPLVQGFVSKL 117
cow             NRETSNPKASNFSFEDPSNKTH-ETGFYSNLSTDNSSRSPSLMPTLSPRSPSTHSFVSKL 119
human           NITTSNLKASHSPPLNLPNNSHGITDFSSNSSAEHSLGSLKPTSTISTSPPLIHSFVSKV 120
                .   **  .:  .  : . .:.  *.   .     *  *    .* *  .*  :.****:


mouse           PLNSSTADANPLQVSEHSNSTNSPSPENFTWSLDNDTMNSPEDISTTVRPFPPPPKTTPV 177
rat             PLNSSVADVNPLQVSEHSNSTHSPLSGNFTWSLDNDTMNSPKDMSSTVSLFPPPPKTTPV 177
cow             PWNSSIADNSLLPASAPPNTTVPVSSENFTLSSINDTMKAPDNSSITVSNLPSGPNTTSV 179
human           PWNAPIADEDLLPISAHPNATPALSSENFTWSLVNDTVKTPDNSSITVSILSSEPTSPSV 180
                *:. ** . *  *  .*:* .  . *** *  ***:::*.: * **  :.. *.:..*


                                                                  ═ ═ ═ :
mouse           TPFTAEPTEWLPTNNDNFAGFTPYQEKTTLQPTLKFTNNSKLFPNTSDTPKENKNTGIVF 237
rat             TPFTAEPNGWFGTN-DNFAGFTPYQEKTTLQPTLKFTNNSKLFPNASDTPKETKNTGIVF 236
cow             TPMVTE--GWPTTTRESMEGFTVYQE-TTLHPTLKFTNNSKIFPNTSDPQEENRNTGVVF 236
human           TPLIVEPSGWLTTNSDSFTGFTPYQEKTTLQPTLKFTNNSKLFPNTSDPQKENRNTGIVF 240
                **: .*   *  *. :.: *** *** ***:**********:***:**. :*.:***:**

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                ═══════════
mouse           GAILGAILGASLLSLVGYLLCGQRKTDSFSHRRLYDDRNEPVLRLDNAPEPYDVNFGNSS 297
rat             GAILGAILGASLLSLVGYLLCGQRKTDSFSHQRLYDDRNEPVLRLDNAPEPYDVNFGNSS 296
cow             GAILGAILGASLLSLVGYLLCGKRKTDSFSHRRLYDDRNEPVLRLDNAPEPYDMSFGNSS 296
human           GAILGAILGVSLLTLVGYLLCGKRKTDSFSHRRLYDDRNEPVLRLDNAPEPYDVSFGNSS 300
                *********.***:********:********:**********************:.*****


mouse           YYNPAVSDSSMPEGGESLQDGIPMDAIPPLRPSI 331
rat             YYNRAVSDSSMPEGGESAHDSIPMDAIPPLRTSM 330
cow             YYNPTANDSSTSAGGENAHDSIPMDDIPPLRTSV 330
human           YYNPTLNDSAMPESEENARDGIPMDDIPPLRTSV 334
                *** : .**: . . *. :*.**** *****.*:
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Fig. 13.11 Multiple alignments of the amino acid sequences of MUC-15. Murine (Q8C6Z1), rat (Q5XHX5), bovine (Q8MI01) and human (Q8N387) MUC-15 were aligned using the ClustalW2 program at the EBI site (http://www.ebi.ac.uk/tools/clustalw2). Consensus symbols denoting the degree of conservation observed in each column are " * ", residues identical in all sequences in the alignment; residues for which conserved " : " or semi-conserved " . " substitutions have been observed. Accession numbers are given in parentheses. *Solid line*: transmembrane region; *dashed line*: missing residues in the alternatively spliced variant (secreted MUC-15, MUC-15/S). *N*-Glycosylation sites are indicated in *bold* (Pallesen et al., 2002)

are due to differences in carbohydrate contents. We showed that BTN either from cow or goat milk does not contain *O*-glycans, but large amounts of (α2.6)-linked sialic acids carried by *N*-linked carbohydrates (Cebo et al., 2009).

13.4.2.2 Lactadherin

The complete amino acid sequence of lactadherin (LDH) are available for bovine (formerly known as PAS 6/7 glycoprotein), human (breast epithelial antigen, BA46), murine (milk fat globule EGF factor 8, MFG-E8), rat and porcine (sperm surface protein P47) proteins (Fig. 13.13). With only a 12-amino acid long sequence located in the C-terminal part of the protein, the full-length sequence for caprine LDH is still missing. Sequence homologies are high across species with values ranging between 61 and 94%. General features of LDH are the presence of two EGF-like domains in the N-terminal part of

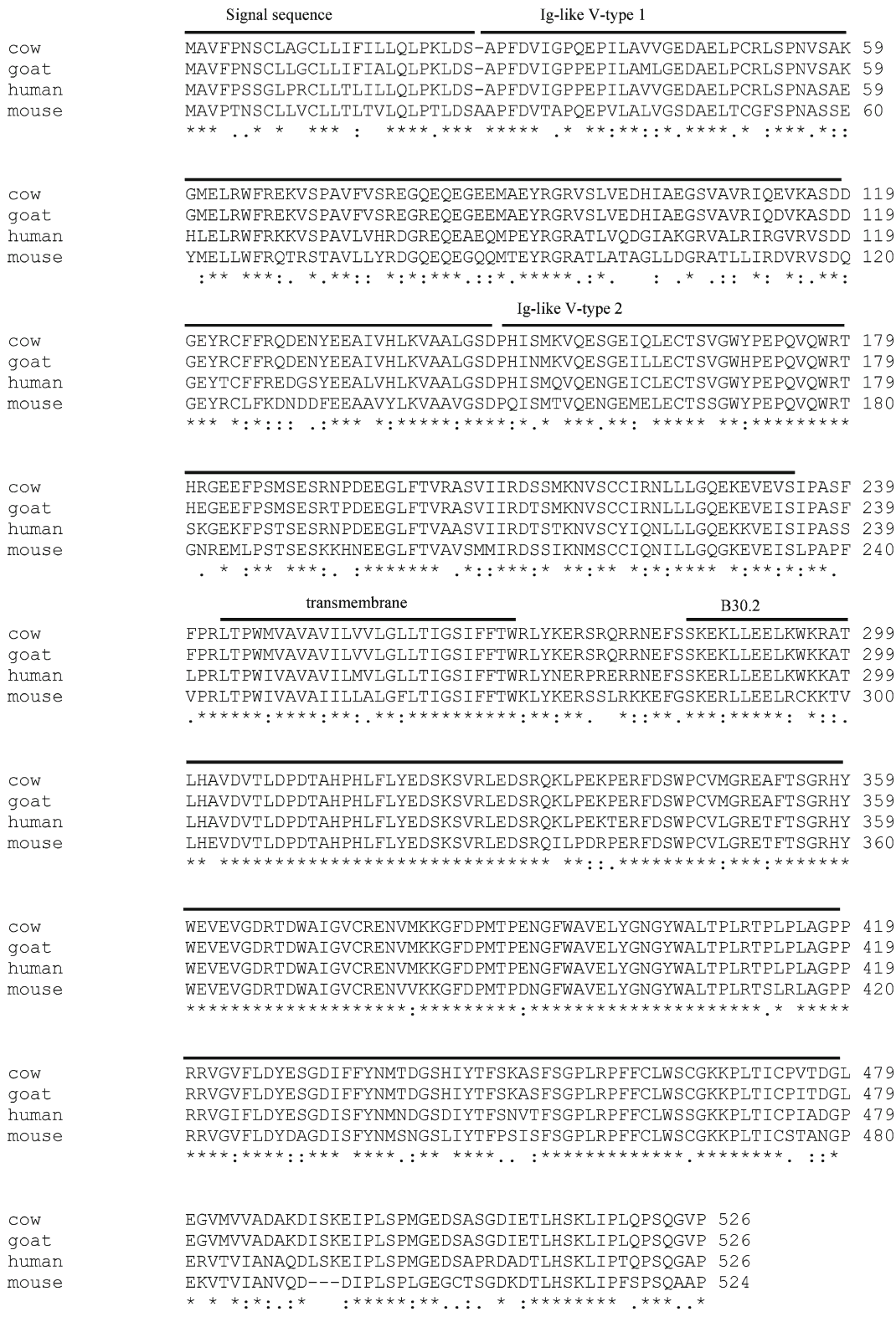

Fig. 13.12 Across species comparison of butyrophilin (BTN) amino acid sequences. Bovine (P18892), caprine (A3EY52), human (Q13410) and murine (Q62556) butyrophilin were aligned using the ClustalW2 program at the EBI site (http://www.ebi.ac.uk/tools/clustalw2). Accession numbers are given in parentheses. Locations of extracellular Ig-like and cytoplasmic B30.2 functional domains are indicated

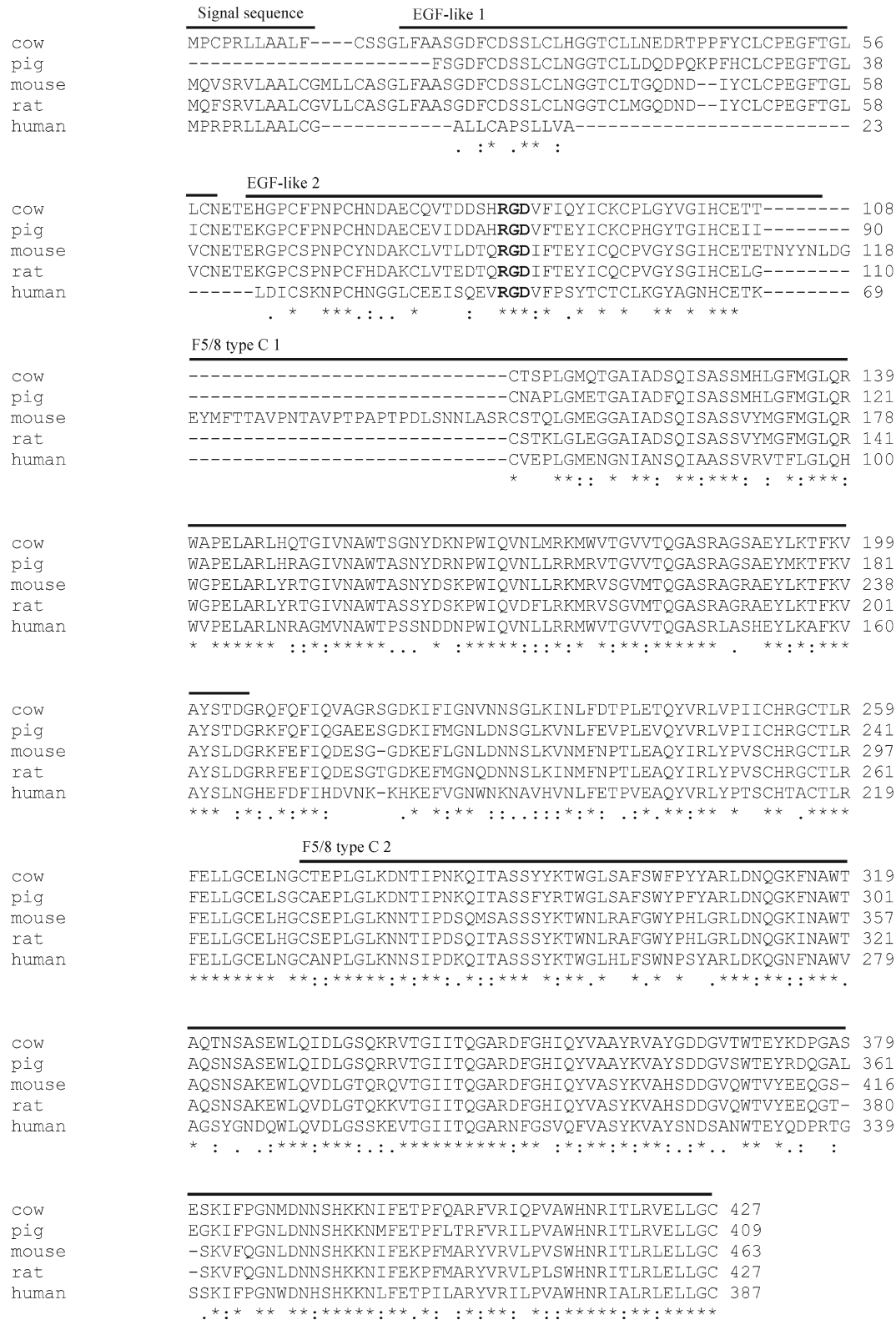

Fig. 13.13 Alignment of the amino acid sequences of bovine (Q95114), porcine (P79385), murine (P21956), rat (P70490) and human (Q08431) lactadherin using the ClustalW2 program at the EBI site (http://www.ebi.ac.uk/tools/clustalw2). Accession numbers are given in parentheses. Locations of EGF-like and F5/8 type C functional domains are indicated. The RGD (Arginine-Glycine-Aspartic acid) adhesive sequence is indicated in *bold*

the protein with an Arginine-Glycine-Aspartic acid (RGD) sequence in the second EGF-like domain, and of two C-like domains of about 150 amino acids called F5/8 type C or C1/C2-like domains also present in coagulation factors V and VIII. The C-terminal domain of the second F5/8 repeat has been shown to be responsible for membrane binding through a phosphatidylserine-binding motif (Foster et al., 1990). The RGD sequence is a cell-adhesion motif able to bind to integrins (Dong et al., 1995). However, some differences have been observed between species. Human LDH, characterised by two proteins of 50 and 30 kDa, does not contain the first EGF-like domain. The 50 kDa protein is the full-length protein also known as breast carcinoma protein BA46 that is highly expressed in human breast tumours. The 30-kDa protein is a truncated form of BA46 consisting of the C-terminal factor V/VIII-like domain which appears to anchor BA46 to the MFGM (Giuffrida et al., 1998). Bovine LDH, also known as PAS 6/7, consists of two polypeptides staining well with Coomassie blue and the PAS reagent. These bands correspond to protein isoforms produced by alternative splicing, a long isoform consisting of 427 amino acids and a short isoform arising from an internal truncation of 52 amino acids starting from position 169 and extending to position 220. This is the consequence of an exon-skipping event occurring during the course of the pre-messengers maturation process (Hvarregaard et al., 1996). Although limited information is available on its sequence, we have shown that LDH from goat milk consists in a single 55-kDa protein by contrast to bovine LDH for which two polypeptide chains of about 52 and 50 kDa in 6% SDS-PAGE are easily identified by peptide mass fingerprinting MALDI-TOF analysis (Cebo et al., 2009). This difference from bovine LDH may be related to a singular secretion mode hypothesised in the goat species (Neveu et al., 2002). In mice, two protein variants produced by alternative splicing of the same premature mRNA have been evidenced: a long 61-kDa isoform, expressed predominantly in the mammary gland, and a short 53-kDa isoform expressed ubiquitously in various tissues including lung, liver, small intestine, testis and mammary gland. The expression of the long variant increases remarkably in late gestation and during lactation. Interestingly, the long variant contains a proline/threonine (Pro-Thr)-rich 37-amino acid domain containing multiple *O*-linked glycans chains which may be functionally important for secretion of milk fat globules from MEC (Oshima et al., 1999). Finally, a new family of zona pellucida-binding proteins homologous to bovine LDH and murine MFG-E8 is growing with the isolation of a 47-kDa protein from porcine sperm. Using antibodies raised against bovine LDH, proteins of apparent molecular mass similar to that of P47 protein were also detected in porcine milk (Ensslin et al., 1998).

13.4.2.3 Adipophilin

Complete amino acids sequences are available for bovine, porcine, human and mouse adipophilin (Fig. 13.14). Sequence similarities are high between species thus suggesting conserved functions for this protein. The N-terminal region of adipophilin contains a sequence motif that is shared by other proteins, namely, perilipin and TIP-47 proteins. They define a new family of lipid droplet-associated proteins called PAT, which is an acronym for perilipin, adipophilin and TIP-47 proteins. Two PAT subdomains are described. The PAT-1 domain (~100 amino acids) defines the high identity N-terminal region, whereas the PAT-2 domain refers to the more distal region of lesser similarity in PAT proteins (Lu et al., 2001; Miura et al., 2002). Interactions between BTN, XOR and adipophilin are supposed to be involved in milk lipid droplet secretion in the MEC (Mather and Keenan 1998). Conversely, the fat content of milk from ADPH null mice was comparable to that of wild-type mice. However, it has been shown that that ADPH null mice display an N-truncated form of adipophilin that retains the ability to promote the secretion of lipid droplets in milk (Russell et al., 2008). Thus, the PAT domain of adipophilin is not directly involved in its physiological function. Levels of transcripts were lower in ADPH null mice than in wild-type mice. By contrast, amounts of ADPH proteins were comparable in mutant

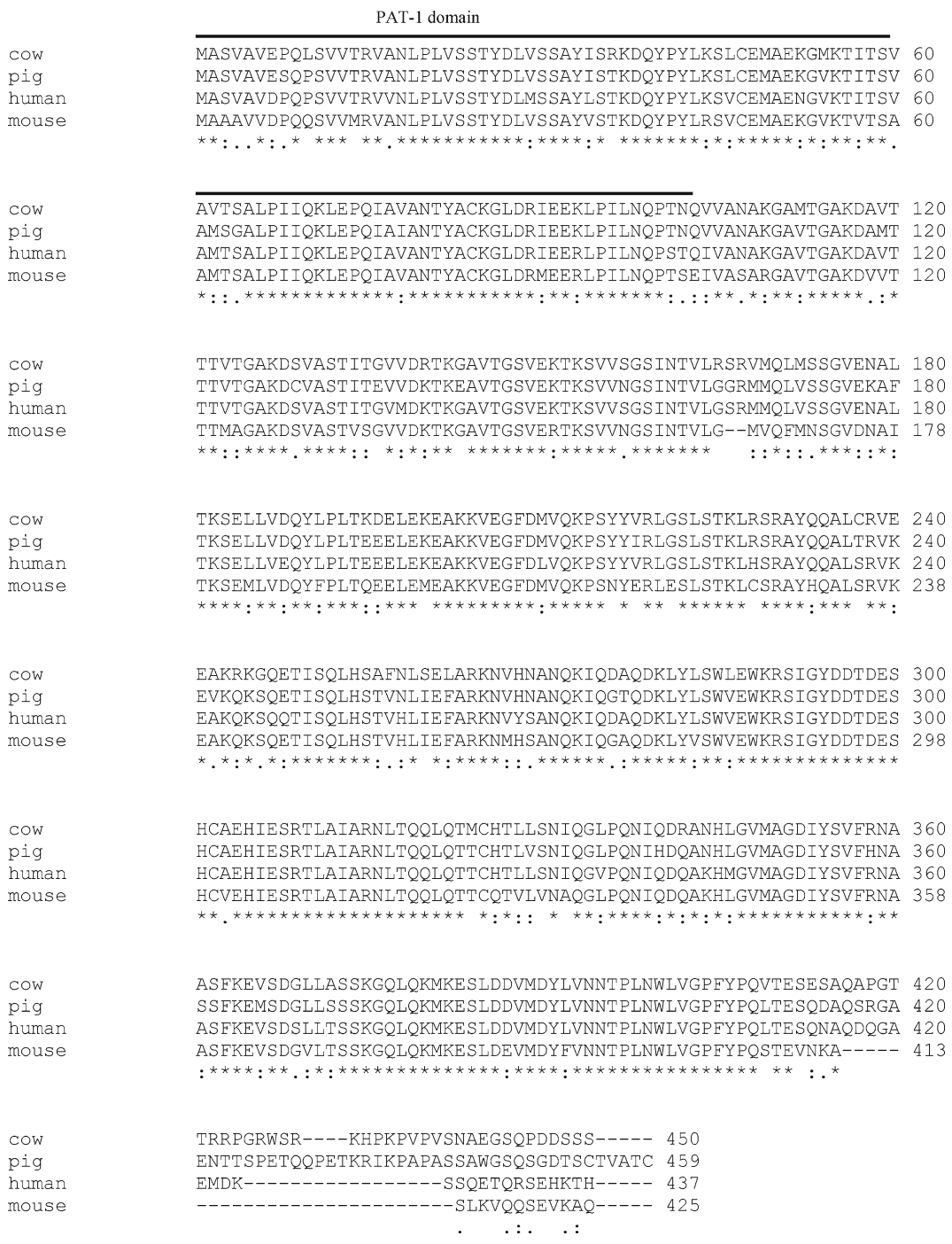

Fig. 13.14 Alignment of the amino acid sequences of bovine (Q9TUM6), porcine (Q4PLW0), human (Q99541) and murine (P43883) adipophilin using the ClustalW2 program at the EBI site (http://www.ebi.ac.uk/tools/clustalw2). Accession numbers are given in parentheses. Location of the PAT-1 domain sharing 40% identity with other murine lipid droplet-associated proteins, namely, perilipin and TIP-47 is indicated. The more distal PAT-2 sequence (~120 amino acids) can also be aligned but more poorly (Lu et al., 2001)

and null mice thus suggesting the N-terminal region of ADPH was involved in the stability of the protein (Orlicky et al., 2008). The PAT region of adipophilin was also shown to control the access of TIP-47 to the cytoplasmic lipid droplet. However, it is not clear if the PAT is directly or indirectly linked to the multiple cellular functions observed (Orlicky et al., 2008).

13.4.3 Glycosylation as a Factor of Variability of MFGM Proteins Through Species

Glycosylation variations through species have been initially reported for MUC-1, most probably because of its high carbohydrate content. Indeed, monosaccharide composition of MUC-1 from bovine milk suggested profound differences with MUC-1 from human milk (Pallesen et al., 2001). A more recent study firmly established the species-dependent nature of carbohydrate structures found in glycoproteins from milk. Glycosylation of MFGM proteins from eight species (human, cow, goat, sheep, pig, horse, dromedary and rabbit) were investigated by using lectins and carbohydrate-specific antibodies (Gustafsson et al., 2005). Large-scale techniques now available (i.e. glycoproteomics) confirmed the differences in the nature of glycans found either on bovine or human MFGM glycoproteins (Wilson et al., 2008). Bovine *O*-linked oligosaccharides were reported to present mono- and disialylated core 1 oligosaccharides (Galβ1-3GalNAc), whilst *O*-glycans from human milk had core type 2 oligosaccharides (Galβ1-3(GlcNAcβ1-6)GalNAc). Interestingly, the Lewis b epitope, which has been shown to be a target for *Helicobacter pylori* bacteria, was present in human but not in bovine MFGM proteins. Most generally, because the extreme diversity of glycans found on MFGM proteins is thought to prevent the attachment of various pathogenic organisms to intestinal mucosa, it may be hypothesised that bovine milk will provide a different protection against pathogens than human milk. A striking demonstration has been recently reported for LDH. It was found that human, but not bovine, LDH inhibits rotavirus infections *in vitro*. Indeed, pre-incubation of virus with LDH significantly reduced infection, whereas pre-incubation of LDH with host cells did not show any effects on rotavirus infection. This suggests that LDH acts as a decoy receptor during the course of infection (Kvistgaard et al., 2004).

Concomitantly to the nature of carbohydrates found on MFGM proteins, a quantitative aspect of health benefits provided by carbohydrates present on milk glycoproteins must be considered. Indeed, a relationship between the variable number of tandem repeats (VNTR), which are domains containing numerous *O*-glycosylation sites, and the resistance to *Helicobacter pylori* infections has already been demonstrated in human for MUC-1 and MUC-6 (Nguyen et al., 2006; Costa et al., 2008). Hence, species showing shorter VNTR regions are supposed to be more susceptible to these bacteria than those presenting larger polymorphic domains, that is, inhibiting in a more efficient way, adhesion of pathogens to host cells. Considering the higher number of tandem repeats found on MUC-1 from human milk compared to MUC-1 from bovine milk, it may explain the lower incidence of infectious diseases in breastfed infants (Schroten 1998).

13.5 Concluding Remarks

The past 10 years have seen a fantastic breakthrough in the knowledge of genome structure and organisation. New insights and clues to better understand mechanistic details involved in the regulation and variability of gene expression have been provided already and are still expected. Data now available on the architecture of the casein locus in several species, including monotremes and marsupials, will contribute to our understanding of the mechanisms responsible for variations and heterogeneity in milk casein composition. However, it is perhaps in the functional field that we might progress significantly in the near future.

Factors of variability of MFGM proteins have been evidenced, both intraspecies (i.e. existence of polymorphisms) and interspecies, with PTM such as glycosylation pointed out as a main factor

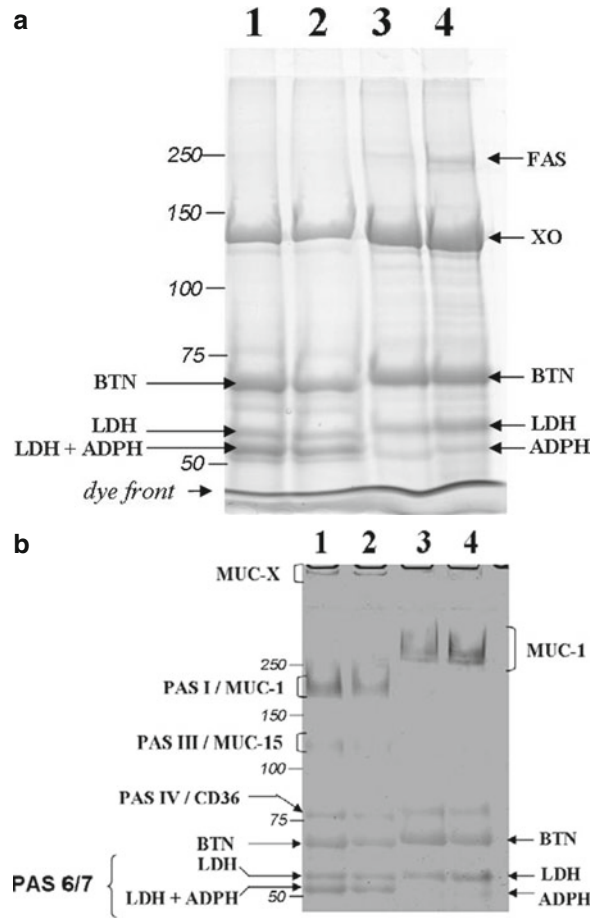

Fig. 13.15 Representative pattern of milk fat globule membrane (MFGM) proteins in SDS-PAGE. MFGM proteins were separated on 6% SDS-PAGE and stained with (**a**) Coomassie or (**b**) PAS reagent (staining of glycoproteins). MFGM proteins are from bovine (*lanes 1–2*) and caprine (*lanes 3–4*) milks. *FAS* fatty acid synthase; *XOR* xanthine oxidoreductase; *BTN* butyrophilin; *LDH* lactadherin; *ADPH* adipophilin. Positions of protein standards (kDa) are indicated to the *left* of the panel (adapted from Cebo et al., 2009)

for the observed molecular diversity of MFGM glycoproteins (Fig. 13.15). Large-scale technologies now available, like proteomics or glycoproteomics, generate a huge amount of data on MFGM proteins. However, efforts need now to be made to go further in the understanding of biological mechanisms underlying this molecular variability.

Development of instrumental techniques has played a key role in these breakthroughs, particularly in the field of PTM. Although we now know many of the details of casein structure, a number of questions remain unanswered in our understanding of the biogenesis of casein micelles. How do caseins interact between themselves and with colloidal calcium phosphate? At what stage are they modified? How is this process influenced by (or influences) the cellular pathway of protein folding and assembly?

Although PTM and genetic polymorphisms were for a long time considered as the most potent factors capable of generating multiple protein products starting from a single gene, it is now obvious that alternative splicing is responsible for a considerable proportion of proteomic complexity in mammals. It is clear that this process must be intimately related to the great diversity and heterogeneity of caseins as well as to their evolutionary pathway. As frameshift mutations, which deeply change the nature of the message and/or lead to premature termination (linked to mRNA decay), such mechanisms, by promoting deletion or addition of a protein domain through exon skipping or cryptic splice site usage, undoubtedly provide a real plasticity to gene information. With at least a total of 21 coding exons that can be, according to the species, constitutively included or skipped, the gene encod-

ing α_{s1}-casein is one of the most impressive examples, in this regard.

Obviously, such a wide structural diversity is unlikely without consequences for the characteristics and the properties of casein micelles, particularly if one considers the possible unique function that seems to be played by α_{s1}-casein in the micelle assembly, transport and secretion (Chanat et al., 1999). In MEC of small ruminants, α_{s1}-casein appears to be a complex mixture of more or less internally deleted proteins. The occurrence of genetic polymorphisms disturbing the splicing machinery adds further to the complexity of the casein fraction. With up to 40 variants of α_{s1}-casein produced in the milk of a single goat, heterozygous A/F at the relevant locus, the secretion pathway may be dramatically disturbed with an impact on milk composition and quality, including modifications in fat structure and composition (Chilliard et al., 2006) as well as in its susceptibility to lipolysis (Lamberet et al., 1996)

Notwithstanding, the growing number of casein genes displaying such complex patterns of splicing, thus increasing the coding capacity of genes, supports the notion that the extreme protein isoform diversity generated from a single gene can no longer be considered as an epiphenomenon. A parsimonious vision of this issue addresses the following question: Does this convey any biological significance? Important new insights are expected, in this field, in the near future.

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Genetics and Biosynthesis of Milk Proteins

14

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14.1 Introduction

During lactation, mammary epithelial cells secrete large quantities of milk proteins. More than 90 % of these proteins are derived from the transcription of a few tissue-specific genes, expression of which is under a complex multi-hormonal regulation that involves both transcriptional and post-transcriptional mechanisms. Furthermore, to fulfil its bioreactor activity, the mammary gland needs an optimal supply of amino acids as well as efficient translation and transport machineries during lactation.

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The previous edition of this chapter (Vilotte *et al.,* 2002) described in detail the hormonal regulation of milk protein gene expression, their mRNA and gene structures, their co- and post-translational modifications and the transport and the secretion of milk proteins. The aim of this revision is to summarize briefly our knowledge on the structure of the milk protein genes and to put into context the rapid growth of information on the regulatory elements involved in controlling the expression of these genes. We will also focus on the amino acid supply to the mammary gland and on the intracellular routing and sorting of milk proteins in mammary cells. However, other important topics will not be discussed. The widespread presence of caseins variants will be covered in Chap. 15, while the practical applications of these studies for the dairy field will be described in Chap. 16. Similarly, global analysis of genome evolution with regard to the mammary gland, as described in Lemay *et al.* (2009), will be discussed in other chapters of this book.

14.2 Structure of Milk Protein Genes

The major milk protein genes have been sequenced in several species. Overall, the mosaic structure of these genes has been well conserved during evolution, and observed species differences in the length of their transcription unit can often be attributed to the occurrence of repetitive DNA within some introns, mainly artiodactyl

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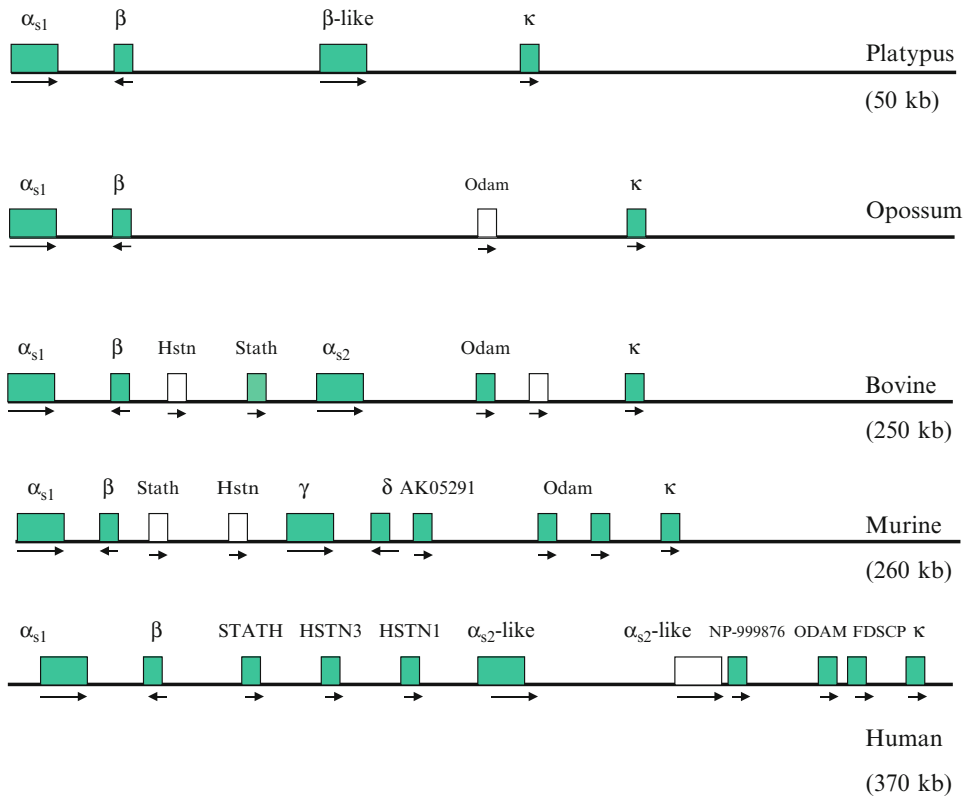

Fig. 14.1 Overall organization of the casein locus in various species. Green boxes represent the transcription unit of the genes. White boxes: putative genes. When available, orientation of the gene transcription unit is indicated by an arrow. Origins of the data are mentioned in the text

retroposons. Similarly, deletions or insertions of amino acids between caseins from different species or variants appear to occur mostly by exon skipping. These genes share the canonical structure of tissue-specific eukaryotic genes. Beyond these basic similarities, these genes differ substantially in their genomic organization.

14.2.1 The Casein-Encoding Genes

14.2.1.1 General Organization of the Gene Cluster

Classical genetic studies have demonstrated that the four casein genes are closely linked, and a general organization of the gene cluster was deduced (reviewed by Grosclaude, 1979). Since then, structural analysis of the gene cluster at the DNA level in various species has confirmed and refined the protein data (Fig. 14.1; Tomlinson *et al*., 1996; George *et al*., 1997; Fujiwara *et al*., 1997; Rijnkels *et al*., 1997a,b,c; 2002; Lefèvre *et al*., 2009). The overall size of the casein locus varies from around 50 kb in opossum to 370 kb in humans, but the order and the orientation of the genes within the cluster are conserved (Fig. 14.1). In mice, the γ- and δ-casein genes, which are linked within 60 kb, encode an α_{s2}-like protein, and sequence analysis suggests that the ancestral α_{s2}-casein gene duplicated at the time of radiation between rodents and Artiodactyla (George *et al*., 1997; Rijnkels *et al*., 1997a). A similar duplication of the α_{s2}-casein gene is also suspected to have occurred in rabbits (Dawson *et al*., 1993). A recent duplication of β-casein was evidenced in the monotreme lineage, and the duplication of the α-casein that occurred in the eutherian lineage is absent in marsupials (Lefèvre *et al*., 2009). Interestingly, the casein locus encompasses several other genes, expression profile of which

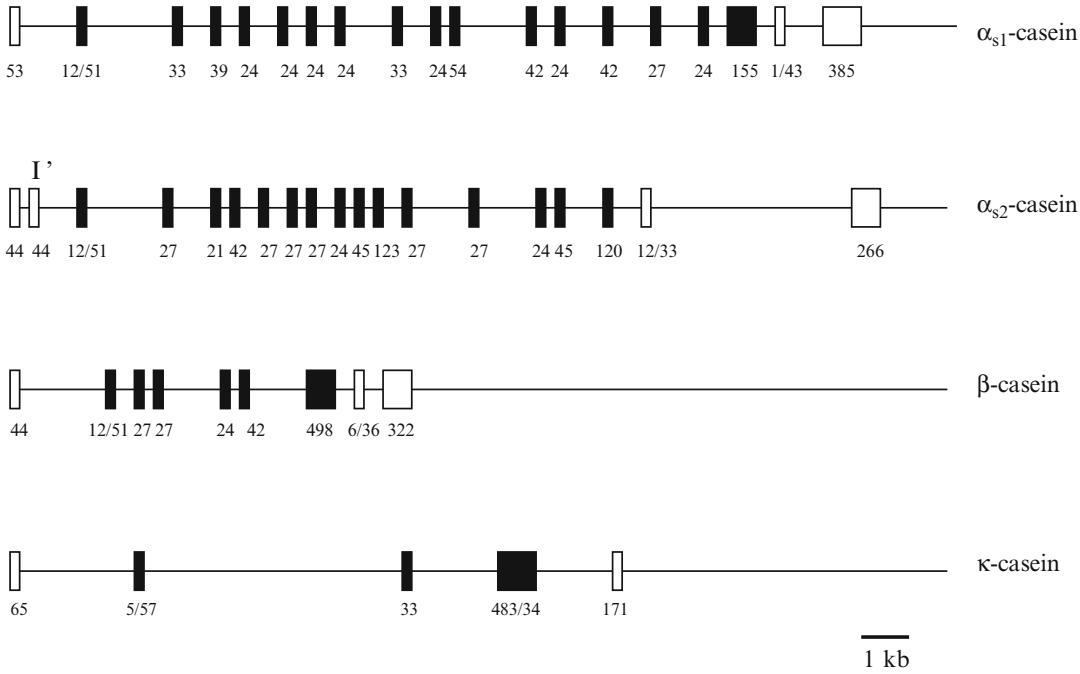

Fig. 14.2 Organization of the bovine casein-encoding genes. Exons are represented by boxes. White boxes: untranslated regions, black boxes: coding frame. Exons are not to scale and their sizes are indicated as base pairs. Two numbers are indicated below exons that comprise both untranslated and coding sequences. Exon I′ from the α_{s2}-casein gene corresponds to a partially skipped exon in the sheep species. Origins of the data are mentioned in the text

may not be restricted to the mammary gland. Inactivation of the β-casein gene (Kumar *et al.*, 1994) did not prevent expression of the remaining caseins within the locus, suggesting that they are expressed independently of each other.

The casein locus has been localized to chromosome 6 in the cow, sheep and goat (Threadgill and Womack, 1990; Hayes *et al.*, 1992; Hayes *et al.*, 1993a; Gallagher *et al.*, 1994); 5 in mice (Geissler *et al.*, 1988); 12 in rabbits (Gellin *et al.*, 1985); 4 in humans; 3 in chimpanzees (McConkey *et al.*, 1996); and 5 in opossum (Lefèvre *et al.*, 2009).

14.2.1.2 Individual Gene Structures

Internal homologies within α_{s1}- and α_{s2}-casein proteins suggested that the cognate genes evolved through intragenic duplications, a result confirmed at the DNA level by the observed duplication of intron-exon-intron stretches. The ubiquity of the major phosphorylation site and the striking homology of signal peptides of α_{s1}-, α_{s2}- and β-casein proteins indicated a possible common origin of these calcium-sensitive casein-encoding genes. This hypothesis was further substantiated by the identification of common sequence motifs in the proximal 5′-flanking region and the similar structural organization of the first four exons. Despite its location within the casein genes cluster, the κ-casein gene appears to have no evolutionary relationship with the calcium-sensitive casein-encoding genes. It was recently hypothesized that the milk casein genes have evolved from genes involved in tooth development before the origin of mammals (Kawasaki *et al.*, 2011).

The structure of the α_{s1}-casein gene has been partially analysed in rat (Yu-Lee *et al.*, 1986) and fully determined in cow (Koczan *et al.*, 1991), goat (Leroux *et al.*, 1992) and rabbit (Jolivet *et al.*, 1992), or could be deduced from several other sequenced genomes, the same being true for the other individual milk protein genes. The transcription unit of the gene is split into 19 exons and spans around 17.5 kb in ruminants (Fig. 14.2).

The structure of the α_{s2}-casein gene has been described in cow (Groenen *et al*., 1993). The gene transcription unit is split into 18 exons and spans 18.5 kb (Fig. 14.2). The first intron contains a non-coding exon (exon I′ in Fig. 14.2) that is known to be retained in 4 % of the ovine mRNA (Boisnard *et al*., 1991). In this species, exon VI is also partially skipped. Sequence comparisons suggest that this gene is more closely related to the β-casein gene than it is to the α_{s1}-casein gene (Groenen *et al*., 1993).

The structure of the β-casein gene is known in mouse (Yoshimura and Oka, 1989), cow (Bonsing *et al*., 1988), rat (Jones *et al*., 1986), rabbit (Thépot *et al*., 1991), goat (Roberts *et al*., 1992), human (Hansson *et al*., 1994) and sheep (Provot *et al*., 1995). Its transcription unit is composed of 9 exons and spans between 8 and 10 kb according to differences between species in the length of intronic sequences (Fig. 14.2). Exon III is skipped in humans, leading to the deletion of 9 amino acids in the mature protein.

Characterisation of the κ-casein gene has been reported in cow (Alexander *et al*., 1988; Kapelinskaia *et al*., 1989), human (Edlund *et al*., 1996) and rabbit (Baranyi *et al*., 1996). The transcription unit of this gene comprises 5 exons, and its length varies from 7.5 kb in rabbit to 12.5 kb in cow (Fig. 14.2). This gene is evolutionarily related to fibrinogens (Jollès *et al*., 1974). Indeed, a 24 bp sequence located at the 5′ end of exon IV of the gene was found to be similar with the end of exon II of the γ-fibrinogen gene, suggesting that it represents an exon from the ancestral gene (Alexander *et al*., 1988).

14.2.2 The Major Whey Protein-Encoding Genes

14.2.2.1 The β-Lactoglobulin-Encoding Gene and Pseudogenes

The structure of the β-lactoglobulin-encoding gene has been reported in sheep (Ali and Clark, 1988; Harris *et al*., 1988), cow (Alexander *et al*., 1993), goat (Folch *et al*., 1994), tammar wallaby (Collet and Joseph, 1995) and horse (Lear *et al*., 1998). The structure of this gene, with seven exons and a transcription unit length of around 4.8 kb, was conserved during evolution (Fig. 14.3). In dogs and horses, two functional and closely linked genes are present in the genome (Halliday *et al*., 1990; Lear *et al*., 1998). In cats, a third functional gene is present (Halliday *et al*., 1990; Pena *et al*., 1999).

In ruminants, a β-lactoglobulin pseudogene has been identified (Passey and MacKinlay, 1995; Folch *et al*., 1996), while in cow and goat a gene conversion event has occurred. The bovine and caprine pseudogenes contain seven exons, and the ancestral protein that they encode is related to the monomeric β-lactoglobulin II protein. The bovine pseudogene is located 14 kb 5′ from the functional gene, in a similar orientation (Passey and MacKinley, 1995).

β-Lactoglobulin belongs to the lipocalin protein family (Flower, 1996, for review). Comparison of the structure of various lipocalin genes with that of the β-lactoglobulin gene has revealed striking similarities, confirming further their evolutionary relationship (Ali and Clark, 1988). The β-lactoglobulin gene(s) and pseudogene were assigned to chromosome 11 in cow and goat, 3 in sheep (Hayes and Petit, 1993c; Folch *et al*., 1996) and 28 in horse (Lear *et al*., 1998).

14.2.2.2 The α-Lactalbumin Gene and Pseudogenes

The α-lactalbumin gene has been sequenced in rat (Quasba and Safaya, 1984), cow (Vilotte *et al*., 1987), human (Hall *et al*., 1987), guinea pig (Laird *et al*., 1988), goat (Vilotte *et al*., 1991), mouse (Vilotte and Soulier, 1992), tammar wallaby (Collet and Joseph, 1995) and otariid and phocid seals (Sharp *et al*., 2008). In eutherians, the gene is composed of four exons and its transcription unit is about 2 kb in length (Fig. 14.3). It shares the same structural organization with the lysozyme gene, corroborating the hypothesis of a common ancestor (Quasba and Safaya, 1984). The structure of the tammar wallaby α-lactalbumin gene appears different with the occurrence of a putative 5′-untranslated first exon (Collet and Joseph, 1995). In ruminants, the occurrence of related sequences, probably pseudogenes, has been reported (Soulier *et al*., 1989; Vilotte *et al*.,

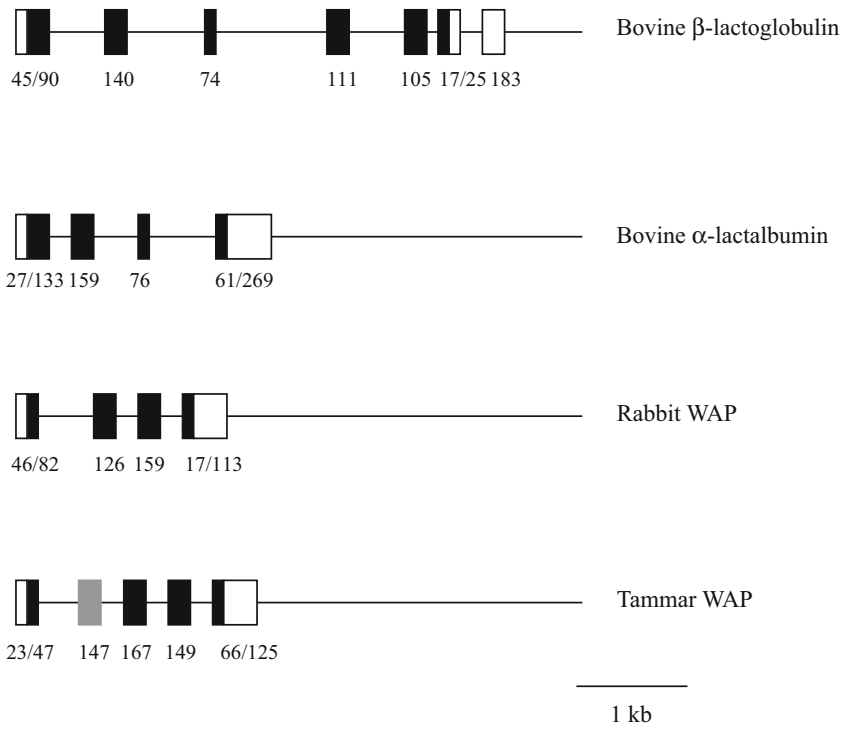

Fig. 14.3 Organization of the major whey protein-encoding genes. Exons are represented by boxes. White boxes : untranslated regions, black boxes: coding frame, grey box: exon 2 from marsupials and monotremes whey acidic protein-encoding gene. Exons are not to scale and their sizes are indicated as base pairs. Two numbers are indicated below exons that comprise both untranslated and coding sequences. Origins of the data are mentioned in the text

1991; 1993). All α-lactalbumin-related sequences are closely linked (Hayes *et al*., 1993b; Gallagher *et al*., 1993) and located 3′ to the functional gene (quoted in Stinnakre *et al*., 1999). The α-lactalbumin-encoding gene (and related sequences) has been assigned to human chromosome 12 (Davies *et al*., 1987), sheep chromosome 3, bovine and goat chromosomes 5 (Hayes *et al*., 1993b) and pig chromosome 5 (Rohrer *et al*., 1997). In Cape fur seals, the gene appears to be transcriptionally silenced and, although comparative analysis of proximal promoter sequence revealed some differences, for which none appears to be responsible (Sharp *et al*., 2008).

14.2.2.3 The Whey Acidic Protein-Encoding Gene

The whey acidic protein (WAP) gene was originally thought to be present only in rodents (Campbell *et al*., 1984) but has been sequenced in various eutherians (Thépot *et al*., 1990; Rival-Gervier *et al*., 2003), marsupials and monotremes (Topcic *et al*., 2009, for review). The 2 kb transcription unit is composed of four exons in eutherians and of five exons in marsupials and monotremes (Fig. 14.3). In ruminants, the WAP gene is characterized by a nucleotide deletion at the end of exon one and a lack of detectable transcription. It thus appeared to be a pseudogene (Hajjoubi *et al*., 2006). The functional role for WAP in milk is unknown, although it bears a similarity to a family of protease inhibitors; however, its absence in deficient mice leads to nutritional deficiencies in the offspring which appear to be unrelated to its activity as a protease inhibitor (Triplett *et al*. 2005). Both WAP and protease inhibitors of the Kunitz family are characterized by highly conserved cysteine residues located in two proteic domains (Hennighausen and Sippel, 1982). The murine WAP gene has been assigned to chromosome 11 (Gupta *et al*., 1982).

14.3 Milk Protein-Encoding Gene Expression and Regulation

14.3.1 Tissue Specificity and Developmental Regulation

The major milk protein genes are defined as mammary-specific and developmentally regulated expressed genes. As such, they represent markers of mammary differentiation. The amount of milk protein mRNA in mammary epithelial cells increases steadily from mid-pregnancy to lactation, although at different rates according to the different genes (Harris *et al*., 1990; Robinson *et al*., 1995). This is due to an increase in the transcription rate of these genes as well as a stabilization of the transcripts (Guyette *et al*., 1979). An observed asynchrony of mammary epithelial cell maturation during pregnancy (Robinson *et al*., 1995) is paralleled by heterogeneous expression during lactation of the major milk protein genes in sheep and cattle (Molenaar *et al*., 1992). This heterogeneous pattern of expression was not observed in lactating mouse mammary glands (Dobie *et al*. 1996). Nevertheless, a short closure of lactating murine mammary gland resulted in local perturbation of milk protein gene expression, leading to the appearance of a mosaic pattern (Faerman *et al*., 1995). These results strongly suggest that the regulation of the expression of the major milk protein genes in mammary epithelial cell is under a complex regulation that could involve both a graded and a binary mechanism.

Over the last few years, the concept that the expression of the milk protein genes is restricted to the mammary gland has been questioned. Expression of α-lactalbumin in the rat epididymis was reported (Qasba *et al*., 1983) and denied (Moore *et al*., 1990; Tang, 1993). The murine α-lactalbumin gene was also reported to be expressed, alongside the β-casein gene, in the sebaceous glands during lactation (Maschio *et al*., 1991), but this observation has not been confirmed (Persuy *et al*., 1992; Vilotte and Soulier, 1992). RT-PCR experiments have suggested that the rat α-lactalbumin gene is also expressed at low levels in the brain of some lactating animals (Fujiwara *et al*., 1999), and casein mRNA expression has been observed in cytotoxic T-lymphocyte-derived cell lines (Grusby *et al*., 1990). Occurrence of casein-like immunoreactive substances in diverse organs, including the thymus, was reported in the rat (Onoda and Inano, 1997). More recently, promiscuous milk protein gene expression was observed in medullary thymic epithelial cells, probably in relation with the development of central T cell tolerance (Derbinski *et al*., 2008). It is of interest to note that many of the tissues where ectopic expression is observed contain some of the transcription factors required for mammary expression of the milk protein genes, for example, in T cells, interleukin-2 activates STAT5 (Gilmour *et al*., 1995).

14.3.2 Hormonal Regulation and Identification of *cis*-Regulatory Elements

Expression of the major milk protein-encoding genes is under a complex multi-hormonal regulation, resulting from the interplay of steroid and polypeptide hormones. In addition, local growth factors and cell-cell and cell-substratum interactions are also involved. Since many reviews have already focused on this topic, including the previous edition of this chapter (Vilotte *et al*., 2002), we will only give a brief survey here.

Schematically, lactogenic hormones, such as insulin, prolactin and glucocorticoids, activate the transcription of the major milk protein genes whereas other hormones, such as progesterone, inhibit this activation in the early stages of pregnancy to favour cell proliferation over cell differentiation. It should be noted that in many systems it is difficult to separate direct induction of milk protein transcription from indirect differentiation-related events. As already mentioned, transcription activation of the major milk protein genes is not concomitant during pregnancy, perhaps due to the presence of various hormonal micro-environments and/or different responses to this environment displayed by different milk protein genes. For example, expression of calcium-sensitive casein genes that are activated at

mid-pregnancy relies on prolactin and is increased by the synergetic action of glucocorticoids (Kabotyanski *et al*., 2006, 2009), while the WAP promoter, a gene expressed late in pregnancy, is reciprocally regulated by these two hormones. In addition, whereas the glucocorticoid induction of the WAP gene expression is rapid, its action on the β-casein gene occurs only with a significant time-lag and requires *de novo* protein synthesis. Expression of the β-lactoglobulin gene, although displaying a similar temporal expression profile to the caseins, appears to be less dependent on lactogenic hormones. Finally, the α-lactalbumin gene, although displaying a temporal expression profile similar to WAP, is induced by prolactin in the presence of low concentrations of glucocorticoids whereas high concentrations of glucocorticoids inhibit its expression, at least in eutherians (Funder, 1989). Induction of the α-lactalbumin gene in the absence of prolactin could be observed in the pregnant murine mammary explants in the presence of insulin and cortisol (Warner *et al*., 1993), while in marsupials, α-lactalbumin gene expression depends only on prolactin (Collet *et al*., 1990). The mechanistic rationale for these intriguing differences has not been identified. Finally, it was recently shown that beside transcriptional regulation and transcript stabilization, lactogenic hormones are also involved in the translational regulation of milk protein synthesis (Rhoads and Grudzien-Nogalska, 2007, for review).

Regulation of mammary gene expression is also controlled by the epithelial cell basement membrane. For example, laminin can induce expression of α-lactalbumin, α_{S1}-casein and β-casein by 160-fold (Aggeler *et al*., 1988; Blum *et al*., 1989). The differences observed between milk protein genes with regard to hormonal induction are also evident in their varying requirement for a basement membrane. Transcriptional control of the β-casein promoter appears less dependent on the three-dimensional structure of the mammary epithelial cells than does the WAP promoter, although both of them are sensitive to extracellular-matrix (ECM) components (Lin *et al*., 1995). At least for the β-lactoglobulin gene, regulation of expression by the ECM occurs through activation of STAT5 (Streuli *et al*., 1995), and this may occur through an ECM-dependent modulation of protein-tyrosine phosphatase activity (Edwards *et al*., 1998). Cell-substratum components as well as glucocorticoids can, at least for the casein genes, also act at the post-transcriptional level (Eisenstein and Rosen, 1988).

Beside these differences, milk protein genes share specific hormonal responses. For example, in most species, progesterone inhibits milk protein gene expression. Although the exact mechanism is still unclear, it has been reported that progesterone might repress expression of the long form of the prolactin receptor mRNA in the mammary gland (Mizoguchi *et al*., 1997) and/or directly repress the prolactin/STAT5-mediated transcription at the milk protein gene promoter level (Buser *et al*., 2007).

Transfection and transgenic studies have revealed that the promoters of most of the major milk protein-encoding genes are responsive to lactogenic hormones sufficiently to target mammary-specific expression of reporter genes in transgenic animals. However, these promoters cannot sustain full expression on their own (compare Webster *et al*., 1995 with Whitelaw *et al*., 1992). Indeed, intragenic sequences of some of the major milk protein-encoding genes were shown to be able to contribute to their hormonal regulation (Lee *et al*., 1989), and important regulatory elements have been identified within the introns (Kang *et al*., 1998, Kolb, 2003) and/or in the 3′ untranslated region (UTR) and flanking regions (Dale *et al*., 1992). Furthermore, and as already mentioned, milk protein genes are also regulated at a post-transcriptional level.

Sequence comparison of a particular gene between several species or from different milk protein genes has led to the identification of conserved DNA motifs that were suspected to be involved in the control of the gene transcription. A classical example is the high conservation of sequence elements in the proximal 5′-flanking region (−200/+1) of the calcium-sensitive casein genes (Rosen, 1987; Kolb, 2002). These early identified elements were subsequently found to

be recognized by regulatory nuclear factors. Consensus sequences recognized by effectors known to be involved in lactogenesis were also identified within the major milk protein gene promoter sequences by computer searches. However, evidence for the presence of a *cis*-regulatory element within a DNA fragment came from transfection experiments in cell cultures, transgenic studies, DNAse I protection, footprinting and/or gel shift assays. Site-directed mutagenesis of the identified binding sites and observations, either in cell culture or in transgenics, of the consequences of such mutations on the promoter transcriptional regulation was sometimes performed to further define the functional role of these elements.

14.3.3 Transcriptional Control of Milk Protein Genes

Binding sites for several transcription factors have been identified within the promoters of most of the major milk protein-encoding genes, such as binding sites for OCT-1, NF-1, C/EBP, STAT5, GR, Ets-1 and YY1 (see Rosen *et al*., 1996, for review). Other DNA elements have been shown to interact with yet unidentified effectors, such as the negative regulatory elements of the WAP promoter (Kolb *et al*., 1994) and of the β-casein promoter (Lee and Oka, 1992; Altiok and Groner, 1993, 1994). Most of these sequences appear to be clustered within short DNA fragments of several hundred base pairs in length that encompassed both positive and negative regulatory elements. Such composite response elements have been identified in the proximal 5′-flanking regions of the β-lactoglobulin gene (region −406/+1; Watson *et al*., 1991), of the calcium-sensitive casein genes (region −200/+1; Rosen *et al*., 1986; 1998 for review), in more distal regions of the bovine (BCE-1 element: region −1613/−1562; Schmidhausser *et al*., 1992, Myers *et al*., 1998) and human (region −4700/−4550; Winklehner-Jennewein *et al*., 1998) β-casein genes, of the rabbit α_{s1}-casein gene (region −3442/−3118; Pierre *et al*., 1994) and of the rat WAP gene (region −949/−720; Li and Rosen 1994a, 1995; Raught *et al*., 1995). A cooperation of distal and proximal promoter elements is required to achieve both maximum expression and maximum hormone responsiveness of the murine β-casein gene in mouse HC11 cells (Robinson and Kolbs, 2009). Thus, it appears that the transcriptional regulation of the major milk protein genes is under a combinatorial control with the binding of multiprotein complexes that can either repress or activate gene expression (see Wolberger, 1998, for review).

Differences in the composition of these composite regulatory elements may explain the observed hormonal differences in the transcriptional developmental regulation between the various major milk protein genes. In the WAP gene, for example, an Ets-1 binding site located at −110 appears to be important for the stage-specific transcriptional activation of the gene but not for its stable expression during lactation (McKnight *et al*., 1995). Activation of promoters by transcription factors can be mediated by the relief of the binding of transcription repressors through both the competitive binding of these activators and the hormonal regulation of the expression or activation of these factors (Schmitt-Ney *et al*., 1991). Thus, some negative binding factors appear to be present in the mammary gland only during pregnancy but not during lactation (Lee and Oka, 1992). Some of them mediate the inhibitory action of progesterone. Similarly, expression of C/EBPβ protein isoforms that are essential both for mammogenesis and lactogenesis (Robinson *et al*., 1998; Seagroves *et al*., 1998) is regulated during pregnancy and lactation. The ratio between LIP, a dominant-negative transcriptional repressor, and LAP, which is an activator of transcription, is high during the pregnancy stage and decreases during lactation due, in part, to the inhibition of LIP expression by glucocorticoids (Raught *et al*., 1995). Expression of C/EBPα is also increased during lactation, possibly following stimulation by some ECM components (Raught *et al*., 1995). The action of another factor, the transcription factor YY1, that represses expression of the β-casein promoter by binding to it at position −120/−110 is counteracted by its replacement from its binding site by the

prolactin-activated STAT5 protein (see below), which in turn positively regulates the promoter activity (Meier and Groner, 1994). A more surprising example of regulation *via* binding of negative regulatory factors is given by the two proteins that bind the upper strand of the β-casein promoter at position −221/−170 during pregnancy and involution (Altiok and Groner, 1994). During lactation, a molecule inhibits the binding of these factors to the gene promoter, and it is suspected that this molecule could be the β-casein mRNA itself that possesses high-affinity binding sites for the two proteins in its 5′ UTR (Altiok and Groner, 1994).

Much has been discovered about how milk protein genes are regulated, and the involvement of many transcription factors has been described. Notwithstanding all this information, the identification of STAT5 as the end point of prolactin signalling in the mammary gland heralded a new era in our understanding of mammary gene regulation. All the more so, since the STAT proteins are central to all cytokine responses (Heim, 1999). Much of the work on STAT proteins has been pioneered by studies involving mammary genes (Schmitt-Ney *et al*., 1991; Watson *et al.,* 1991).

14.3.4 Prolactin Signal Transduction

In the mammary gland, prolactin induction results in the expression of the milk protein genes. This occurs through a rapid but transient signalling transduction pathway (Heim, 1999). First, prolactin-induced dimerisation of its receptor causes *trans*-phosphorylation of the kinase which is constitutively associated with the cytoplasmic domain of the receptor. The kinase is called JAK2 (for janus kinase 2). The activated JAK2 phosphorylates the receptor creating a docking site for STAT5 through its SH2 domain. Subsequent phosphorylation and dimerisation of STAT5 result in its translocation to the nucleus where it binds to GAS (γ-interferon activation sequences) elements in target genes, for example, β-lactoglobulin. Many other proteins are associated with this pathway, for example, MAPK and SOCS, resulting in a logistical problem for the cell to manage if it is to maintain tight regulation of the signal. It does this by balancing the activation signal with the generation of factors that inhibit the signal (Starr and Hilton, 1999; Tomic *et al*., 1999).

STAT5 is an important positive transcription factor in the transcriptional regulation of milk protein genes (see Barash, 2006, for review). It is involved in the transduction of the prolactin signal. Functional STAT5 binding sites have been identified in the promoter region of almost all major milk protein-encoding genes, with the potential exception of the κ-casein-encoding gene (Adachi *et al*., 1996). Within calcium-sensitive casein and β-lactoglobulin composite elements, the occurrence of multiple STAT5 binding sites is observed. These STAT5 binding sites were shown to be essential to confer prolactin transcriptional stimulation to the linked promoter (Schmitt-Ney *et al*., 1991; Demmer *et al*., 1995; Jolivet *et al*., 1996; Soulier *et al*., 1999). Furthermore, several experiments suggest that STAT5 effects are limited to the modulation of expression level, but are not involved in determining the tissue specificity of expression. For example, mutation of one or several of the STAT5 binding sites within the β-lactoglobulin promoter did not affect the tissue-specific expression of this gene in transgenic mice (Burdon *et al*., 1994).

The STAT5 transcription factor actually consists of two proteins, STAT5a and STAT5b. These highly similar proteins are encoded by two genes, with differences in their binding affinities due to a single amino acid substitution (Boucheron *et al*., 1998). The role of these proteins has been studied using gene knockout approaches, with STAT5a emerging as the major factor required for milk protein gene expression. STAT5a-deficient mice exhibit defective mammary gland development (Liu *et al*., 1997). As one might expect, given the different response of the various milk protein genes to hormones, STAT5a knockout mice express the milk protein genes to different levels. Essentially normal levels of β-casein and α-lactalbumin mRNA levels are detected, with only WAP mRNA levels showing

a reduction (Liu *et al*., 1997). Indeed, there is no correlation between the expression of the STAT5a and the β-casein genes (Kazansky *et al*., 1995). Studies with STAT5b knockout mice indicate that this protein is associated with growth hormone effects (Udy *et al*., 1997).

Altogether, these observations suggested that STAT5 may facilitate the interaction of other transcription factors to allow the transcriptional activation of the promoter. Indeed, functional interaction between STAT5 and the glucocorticoid receptor was reported, and this molecular complex was demonstrated to cooperate in the induction of the β-casein promoter, independently of the DNA-binding function of the glucocorticoid receptor (Stöcklin *et al*., 1996, 1997; Lechner *et al*., 1997).

Better understanding of the nature and structural arrangement of the *cis*-regulatory elements involved in the control of the transcription of the major milk protein genes has given an insight into the differential developmental regulation of their expression. However, many questions remain. Why is expression of WAP in preference to β-casein affected in the STAT5a-null mice given that the reciprocal requirement for prolactin is observed? If it is not STAT5, and it appears not to be, what confers mammary specificity to milk protein gene expression? How do the various transcription factors involved interact with each other to generate a production transcription complex? How does this transcription complex interact with the underlying nucleosomes?

14.3.5 Milk Protein Gene Chromatin Domains

It is now generally accepted that chromatin, or its epigenetic regulation, plays a central role in the regulation of gene expression (Rijnkels *et al*., 2010). Studies on the ovine β-lactoglobulin gene brought indirect, although compelling, evidence that chromatin reorganization is important for milk protein gene expression. Concomitant to the increase in β-lactoglobulin gene expression during pregnancy, due to activation of STAT5, changes in DNase I hypersensitivity in the β-lactoglobulin gene were reported to occur (Whitelaw and Webster, 1998). This implies a reorganization of the chromatin in response to the interaction of transcription factors. Furthermore, this assay has detected species-specific differences in this reorganization of the chromatin (Pena *et al*., 1998). These differences are important clues to understand the various levels of expression of the same gene between species or between alleles (Whitelaw, 2000). Similar studies have shown that chromatin reorganization events also occur during the induction of the expression of the rat WAP gene (Li and Rosen, 1994b). However, the clearest evidence yet describing a role for chromatin structure in the regulation of milk protein gene expression comes from the analysis of the β-casein BCE-1 element. This element is not activated in transient transfections, in which true chromatin is not formed, and is responsive to the state of acetylation of the histones (Myers *et al*., 1998).

The transcription domains of the major milk protein genes are poorly defined. As yet, they have generally been defined through functional analyses only. Transgenic studies have revealed that while the α_{s1}- and the β-casein genes from various species can be expressed at relatively high levels in mice, only weak expression of the α_{s2}- and the κ-casein transgenes was observed. It is thus hypothesized that a mammary-specific locus control region (LCR) might control the expression of the casein locus and that this putative element is located close to the α_{s1}- and the β-casein loci. So far, however, no direct evidence for the existence of such an element has been provided. Furthermore, as the casein gene clusters of some species contain several genes which are not expressed tissue specifically in the mammary gland (Fig. 14.1), the influence of a putative LCR would have to be selective for the casein genes.

A consequence of chromatin gene domains is that boundaries to these domains must exist. There are several documented examples (see Geyer, 1997, for review), but again, as yet, none has been conclusively identified for a milk protein gene. However, some predictions are possible using genome comparison. The boundaries of the α-lactalbumin gene are not known, but those of the related lysozyme gene are well defined (Phi-Van

and Strätling, 1988). It is likely that the α-lactalbumin gene contains similar elements, probably in a similar location. This hypothesis is supported by the observed position-independent and copy number-related expression of a human 250 kb YAC and of a 160 kb goat BAC-α-lactalbumin transgene (Fujiwara *et al*., 1997; Stinnakre *et al*., 1999).

At present, the transcription domain of the β-lactoglobulin gene might be one of the best studied within milk protein genes. Several DNase I-hypersensitive sites that reflect its expression status have been located within the promoter, intronic and 3′-flanking regions (Whitelaw and Webster, 1998). These sites spatially reflect the limit of the chromatin domain, as defined by nuclease sensitivity. The 5′ limit of this domain resides very close to the promoter. In addition, the proximal 3′-flanking β-lactoglobulin sequences can interact with the nuclear matrix in vitro. This suggests that the β-lactoglobulin gene resides in a very small chromatin domain (Whitelaw, unpublished results). Sequences that interact with the nuclear matrix, usually AT-rich in nature, may be involved in regulating chromatin structure thereby facilitating gene expression. Although several studies have addressed the ability of such sequences to enhance milk protein gene expression, a clear picture has not yet emerged (e.g., Attal *et al*., 1995; McKnight *et al*., 1996). Another well-studied locus is that encompassing the WAP gene, the DNA of which adopts different chromatin loop structures according to the studied tissue and developmental stage that allow the differential expression of the genes it contains (Montazer-Torbati *et al*., 2008) and regulation from distant enhancers and/or repressors (Saidi *et al*., 2007).

14.3.6 Milk Protein mRNAs

The structure of milk protein mRNAs has been investigated in numerous species (see Mercier and Vilotte, 1993, for review). In lactation, these RNAs account for up to 60 %–80 % of the total RNA present in mammary epithelial cells. The calcium-sensitive casein mRNAs are characterized by a better interspecies conservation of the UTRs and of the sequence encoding the signal peptide compared to the mature protein-coding frame. Since part of the regulation of milk protein gene regulation is exerted at a post-transcriptional level (see Aggeler *et al*., 1988; Blum *et al*., 1989; Golden and Rillema, 1995, for examples), conservation of the UTR might reflect their importance for the mRNA processing. In many cases, the rate of translation is influenced by the 5′ UTR sequence and its secondary structure. Sequences located in the 3′ UTR are also known to interact with proteins to either stabilize or degrade the RNA, and/or alter mRNA translation efficiency, and to be potential targets for miRNA. Involvement of miRNA in the biology of the mammary gland is still poorly documented. However, recent studies suggested their implication during normal mammary gland development and differentiation (Gu *et al*., 2007; Wang and Li, 2007; Avril-Sassen *et al*., 2009; Sdassi *et al*., 2009; Tanaka *et al*., 2009; Ucar *et al*., 2010). A direct role for miRNA in the synthesis of a milk protein was recently evidenced with the identification of a conserved region in the 3′ UTR of the lactoferrin gene targeted by miR-214 (Liao *et al*., 2010).

As already mentioned, total or partial exon skipping during the splicing of the pre-mRNA is responsible for the differences observed between casein variants and between homologous proteins from different species. In this section, we will just describe variations that affect the overall level of gene expression. Deletion or transition of a single nucleotide within coding exons of a caprine α_{s1}-casein allele and of two β-casein alleles creates nonsense codons (Leroux *et al*., 1992; Persuy *et al*., 1996; Rando *et al*., 1996). These alleles are characterized by a much lower level of mRNAs compared to other alleles and, for some of them, with multiple exon-skipping events. This phenomenon has been observed for several other genes where nonsense codons have been found to be associated with mRNA decay and/or exon skipping (see Valentine, 1998; Hentze and Kulozik, 1999, for reviews). Goat and bovine α_{s1}-casein alleles, also associated with reduced amount of mRNA and milk protein, were found to contain a truncated inserted LINE element in the 3′UTR (Perez *et al*., 1994; Rando *et al*., 1998). Insertion of these elements is supposed to reduce mRNA stability.

14.4 Milk Protein Synthesis and Secretion

Secretion of milk protein is obviously of interest due to its physiological and economic importance but also from the point of view of the study of high-efficiency secretory pathways. The trafficking and processing events leading to the secretion of milk proteins are known in general outline, but relatively little is established of the molecular cell biology of milk protein transport in the secretory pathway of mammary epithelial cells.

14.4.1 Morphological Organization of the Mammary Secretory Epithelium

Functional differentiation of the mammary gland is linked to the development of its epithelial tissues. From the onset of pregnancy, the duct cells enter a proliferation and differentiation period leading to the development of a highly branched ductal tree which fills the entire mammary fat pad. Alveolar structures, or acini, develop at the ends of the side branches, and terminal differentiation of the alveolar mammary epithelial cells is completed at the end of gestation with the start of milk secretion at parturition. Functional acini are embedded in a stroma composed of connective and adipose tissues, fibroblasts, plasma cells, blood vessels and nerve terminals. These morphological aspects are well documented at http://mammary.nih.gov/

The acini consist of a single layer of cuboidal mammary epithelial secretory cells sealed together at the luminal border by tight junctions so that passage of molecules from the interstitial space to the lumen of the acini by a paracellular route is restricted during lactation, confining such molecular movements to the cellular trans-cytotic pathway. Progenitor cells are believed to reside below the monolayer of luminal cells. Contractile myoepithelial cells that have long spidery processes embrace the alveoli secretory cells. They participate in milk ejection by squirting milk out of the acini lumen into the ducts. Finally, these cells that constitute the acini are separated from the interstitial space by a basement membrane like in any epithelial tissue.

14.4.2 The Functional Compartmentalisation of the Biosynthetic-Secretory Pathway of Mammary Epithelial Cells

During lactation, mammary epithelial cells are highly polarized and display features typical of cells specialized for secretion (Bargmann and Knoop, 1959; Wooding, 1977; Pitelka and Hamamoto, 1983; illustrated in Figs. 14.4 and 14.5). Their cytoplasm contains an extended network of numerous parallel lamellar cisternae and branching tubules decorated with electron-dense ribosomal particles: the rough endoplasmic reticulum (ER). As described in other polarized epithelial cells, the Golgi apparatus is typically located in the peri- and supranuclear region of the cell, close to the centrosome. The Golgi cisternae are well developed, more or less distended, often containing electron-dense particles and filamentous materials. Of note, unusually large vesicles are observed on the *trans* side of the Golgi apparatus. It is not clear whether these dilated structures constitute the *trans*-Golgi network (TGN) or represent newly formed secretory vesicles *en route* to the apical plasma membrane (APM). In addition, numerous smaller vesicles, some of them coated with the typical spike structure characteristic of clathrin, are associated with the *trans* side of the Golgi. Secretory vesicles that have pinched off from the TGN contain filamentous structures and casein micelles similar to those found in milk, in an electron-lucent fluid.

14.4.3 Intracellular Transport and Co- and Post-translational Modifications of Milk Proteins

In mammary epithelial cells, transport of newly synthesized proteins destined for secretion proceeds according to the general biosynthetic-secre-

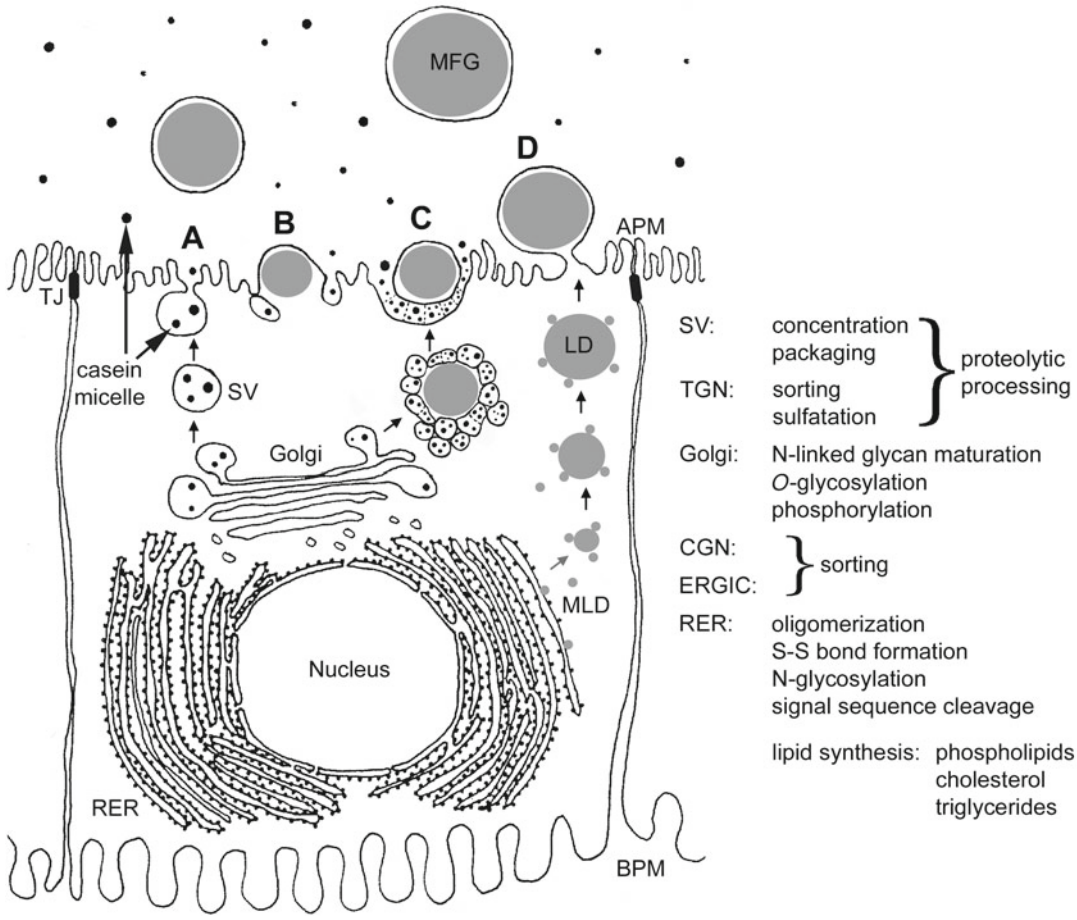

Fig. 14.4 The intracellular compartments of the mammary epithelial cell involved in the biosynthesis and secretion of milk proteins and lipids. Milk proteins undergo various co- and post-translational modifications during their translocation into the lumen of the rough endoplasmic reticulum (RER) and further transport to the apical cell surface, *via* the Golgi apparatus. Casein self-association starts in the lumen of the RER and proceeds into long loose linear aggregates in the *trans*-Golgi compartments and early secretory vesicles (SV). They further self-associate before release, and structures with the characteristic morphological aspect of casein micelles are found in distal SV (see Fig 14.5). Casein-containing SV reach and fuse with the apical plasma membrane (APM) by exocytosis (pathway A). Microlipid droplets (MLDs) emerge from the RER and may fuse with each other and with larger cytoplasmic lipid droplets (CLDs) as they are transported to the apex of the mammary epithelial cells. CLDs and caseins secretion may be partly (pathway B) or significantly (pathway C) coupled (dotted lines indicate that membrane of the casein vesicles has fused together) since the budding of the CLDs requires considerable amounts of APM. Pathway B may only involve heterotypic fusion between the casein vesicles and the APM while pathway C may imply both homotypic between SVs and heterotypic fusion of SVs with the APM. Alternatively, CLDs may be secreted independently of casein-containing SV (pathway D), and some MLDs may also be directly secreted at the apical side of the MECs (not shown). Basal plasma membrane (BPM), *cis*-Golgi network (CGN), ER-Golgi intermediate compartment (ERGIC), lipid droplet (LD), milk fat globule (MFG), tight junction (TJ), *trans*-Golgi network (TGN). Redrawn from Chat *et al.* (2011) (Courtesy of S. Truchet)

tory pathway (Palade, 1975). Following a 3-min pulse labelling with a radioactive amino acid, newly synthesized proteins are detectable in the ER 5 min after the beginning of the pulse, concentrated in the Golgi region after 15 min, accumulated in apical secretory vesicles after 45 min and are predominantly located in the lumen of the acini after 60 min (Seddiki and Ollivier-Bousquet, 1991).

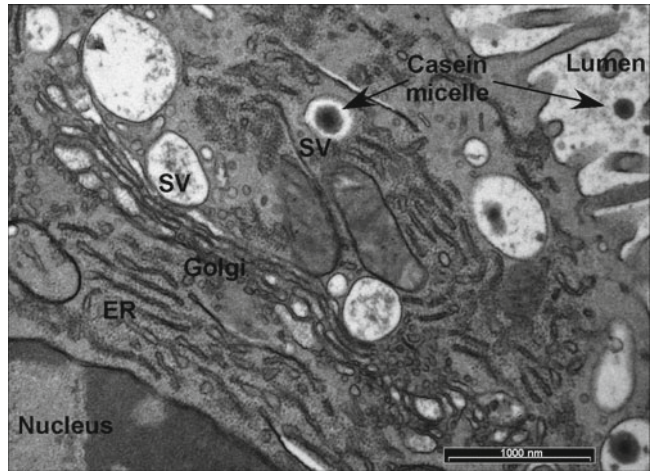

Fig. 14.5 Transmission electron micrograph of part of a mouse mammary epithelial cell during lactation. The major compartments of the secretory pathway are illustrated. Filamentous material is found in dilated Golgi cisternae and in secretory vesicles (SV) close to the *trans*-Golgi, whereas more condensated protein aggregates and typical casein micelles are present in more distal SV and in the lumen of the acini. ER, endoplasmic reticulum (Courtesy of S. Chat and C. Longin)

14.4.3.1 Translocation into and Transport from the Endoplasmic Reticulum

Milk proteins are of two types: transmembrane proteins and water-soluble proteins. An example of milk transmembrane protein is butyrophilin which is first targeted to the plasma membrane from which it is released into milk as part of the milk fat globule (MFG) membrane. The vast majority of milk proteins, however, are water-soluble proteins, for example, the caseins. Entry into the ER requires a signal sequence (Blobel and Dobberstein, 1975). Translocation of milk protein polypeptide chains into the ER lumen and subsequent cleavage of their signal sequence involve classical mechanisms (Schatz and Dobberstein, 1996).

Once translocated into the ER lumen, proteins are in an oxidizing environment which promotes the formation of disulfide bonds between cysteine residues. Most proteins synthesized in the rough ER are glycoproteins. They are modified by a common oligosaccharide on target asparagines residues (*N*-glycosylation). *N*-linked oligosaccharides are added co-translationally and serve as tags to monitor the state of protein folding. The proper folding of proteins that are made in the ER is assisted by proteins with chaperone activities, including BiP/GRP78, Erp77, calnexine and calreticulin (Pelham, 1989). These ER-resident proteins bind to misfolded newly synthesized proteins and retain them in the ER until they have achieved their properly folded or oligomeric state. Correct protein folding or oligomerisation is prerequisite for protein export from the ER and transport to the Golgi complex. This process has been named "quality control" (Hammond and Hellenius, 1995). Accumulation of improperly folded proteins in the ER causes a stress which triggers a coordinated adaptive programme called the unfolded protein response (UPR).

Elucidation of the secondary structure of α-lactalbumin and β-lactoglobulin has revealed the presence of four and two disulfide bridges, respectively (Brew *et al*., 1970; Papiz *et al*., 1986). As to WAP, it contains numerous cysteine residues which form multiple intramolecular disulfide bonds (Hennighausen and Sippel, 1982, Devinoy *et al*., 1988). Moreover, native WAP is dimeric (Baranyi *et al*., 1995). Analysis of the primary structure of κ-casein reveals the very high degree of conservation of at least one cysteine residue in the N-terminal domain of the protein (Bouguyon *et al*., 2006). Dimers of

κ-casein were found in all milk studied, and interchain disulfide bridges were also found for any casein possessing a cysteine residue (Bouguyon *et al*., 2006), for example, in mouse and rat which express four or five cysteine-containing caseins, respectively. As expected, disulphide bond formation between casein molecules was demonstrated to occur within the ER lumen (Le Parc *et al*., 2010). Finally, *N*-glycosylation of rat α-lactalbumin has been detected in mammary microsomal membranes (Lingappa *et al*., 1978). Although *N*-glycosylated forms of α-lactalbumin have been observed in several species, only rat α-lactalbumin is efficiently glycosylated (Prasad *et al*., 1979; Chanat, 2006).

Caseins are not *N*-glycosylated and were shown to lack appreciable amount of regular secondary structure. β- and κ-caseins might possess premolten or molten globule conformations whereas α_{S1}- and α_{S2}-caseins are intrinsically unstructured proteins (Farrell *et al*., 2006; see Chap. 5) or natively unfolded proteins (Holt and Sawyer, 1993). The characteristic structural feature of natively unfolded proteins is a combination of low mean hydrophobicity and relatively high proportion of charged residues at physiological pH (Uversky *et al*., 2000). Proteins with such an open structure possess a peculiar aggregative behaviour and are prone to interact with their specific ligand in vivo. The caseins, however, do not fulfil these two criteria since they present relatively high hydrophobicities.

To date, the question whether caseins are subjected to the ER quality control machinery has not been directly addressed. Moreover, whether or not soluble luminal cargo proteins need to be concentrated and/or require intrinsic sorting information for loading into COPII-coated transport carriers at the ER exit sites to move to the Golgi apparatus is still controversial (for review see Lee *et al*., 2004). Some secretory proteins seem to enter a transport carrier without positive selection by a signal-independent mechanism known as "bulk flow" (Rothman and Wieland, 1996). Finally, as stated above, subunit oligomerisation is required for export of multimeric proteins from the ER (Copeland *et al*., 1986; Kim and Arvan, 1991). Whether interaction between at least some of the caseins in the ER lumen is a prerequisite for their forward transport to the Golgi apparatus is not clear. However, investigation of the impact of the polymorphism at the α_{s1}-casein locus on goat milk secretion has shown that, in the absence of α_{S1}-casein, other caseins accumulate in the ER (Chanat *et al*., 1999). The efficiency of casein transport from the ER to the Golgi apparatus was strongly affected in this context. Data suggested that interaction of caseins in a yet-to-be-identified structure including α_{S1}-casein is required for efficient transport of these proteins to the Golgi apparatus. More recently, the existence of a membrane-associated form of α_{S1}-casein in the ER and more distal compartments of the secretory pathway of mammary epithelial cells has been reported (Le Parc *et al*., 2010), further suggesting a key role of α_{S1}-casein in casein transport in the biosynthetic pathway.

From the above considerations, it is now obvious that first interactions between the caseins take place in the ER and that the exit of casein from this compartment is a key step in casein micelle biogenesis and casein transport in the secretory pathway.

14.4.3.2 Transport Through the Golgi Apparatus

Protein transport through the Golgi apparatus may occur according to the vesicular transport model (stable Golgi cisternae) or the cisternal maturation model (Glick and Malhotra, 1998). Many lines of evidence now support this later model in which the Golgi cisternae themselves move through the Golgi stack. The observation that electron-dense structures, most likely casein aggregates, are detectable in Golgi cisternae of lactating mammary epithelial cells but are excluded from Golgi-associated vesicles is in agreement with this cisternal maturation model (Clermont *et al*., 1993).

Golgi enzymes carry out protein modifications that include glycosylation, phosphorylation, sulphation and proteolytic processing (Fig. 14.4). In mammary epithelial cells, glycosyltransferases are notably involved in the *O*-glycosylation of κ-casein. Moreover, galactosyltransferase, with the help of α-lactalbumin, is also responsible for

the synthesis of lactose (Ebner and Brodbeck, 1968). Galactosyltransferase activity has been detected within the Golgi apparatus but also in secretory vesicles and milk (Witsell *et al*., 1990; Boisgard and Chanat, 2000). The synthesis of lactose in the *trans*-most cisternae of the Golgi apparatus, and most likely in secretory vesicles, surely explains the swollen aspect of these organelles in mammary epithelial cells. The striking reduction of the volume of casein-containing secretory vesicles in α-lactalbumin-deficient mice is in agreement with this hypothesis (Stinnakre *et al*., 1994). Phosphorylation of the calcium-sensitive caseins on serine clusters allows calcium phosphate binding and further interactions between caseins. Like in other cell systems, the kinases that phosphorylate the caseins are located within the Golgi apparatus (Bingham and Farrell, 1974; West and Clegg, 1984). Notably, the phosphorylation of β-casein seems delayed compared to that of α_{S1}-casein (Turner *et al*., 1993; Boisgard and Chanat, 2000; Péchoux *et al*., 2005). This suggests that strong interaction of β-casein with casein polymers might be postponed until it is trafficked to *trans*-Golgi cisternae (see below). Protein sulphation is a ubiquitous TGN-specific post-translational modification. Beside sulphated proteoglycans (Boisgard *et al*., 1999), the sulphation of both α-lactalbumin and κ-casein from rat has been described (Chanat, 2006). One the other hand, although no direct evidence for the cleavage of milk proteins by endoproteases has been reported, furin has been shown to be relatively abundant in Golgi-derived clathrin-coated vesicles from lactating rabbit mammary epithelial cells (Pauloin *et al*., 1999).

14.4.3.3 Transport from the *trans*-Golgi Network and Secretory Vesicle Exocytosis

Secretory proteins are segregated, highly concentrated and packaged into appropriate transport vesicles in the TGN. Sorting and concentration are believed to involve the selective aggregation of the secretory proteins in the ionic environment of the TGN (Chanat and Huttner, 1991), as well as retrieval of excess membrane and luminal content present in newly formed secretory vesicles in clathrin-coated vesicles (Tooze, 1998).

Pre-micellar aggregates have been observed by electron microscopy in the lumen of the Golgi cisternae (Clermont *et al*., 1993). Consistent with the presence of a high calcium concentration in the *trans*-most Golgi cisternae (Neville and Watters, 1983), there is a drastic rearrangement of the micellar structure during the formation of secretory vesicles at the TGN and their transport to the APM for exocytosis. In newly formed transport vesicles, caseins are concentrated in the form of long loose linear aggregates (Mather and Keenan, 1983; Clermont *et al*., 1993). These progressively self-associate and become bigger and denser, and structures with the characteristic honeycomb texture of casein micelles from milk are found in distal secretory vesicles (see Fig. 14.5). The mean size of casein micelles varies widely across species, and the relative proportion of κ-casein was demonstrated to be a modulator of micelle size (Gutierrez-Adan *et al*., 1996). Noteworthy, casein micelles, or at least big aggregates, still form in mammary epithelial cells from of α_{S1}-, β- or κ-casein-deficient animals (Kumar *et al*., 1994; Chanat *et al*., 1999; Shekar *et al*., 2006). Interactions between the various caseins and minerals during micelle biogenesis in the secretory pathway might therefore involve rather general physico-chemical and biochemical characteristics of these components. However, these characteristics are specific enough to avoid incorporation of whey proteins in the micelles. On the other hand, casein-containing secretory vesicles might undergo maturation before exocytosis (Pauloin *et al*., 1999). Finally, reports support the notion that secretory vesicles destined to fuse with the apical cell surface transport both caseins and whey proteins (Devinoy *et al*., 1995; Neville *et al*., 1998; Ollivier-Bousquet, unpublished observation).

A large body of evidence supports the concept that local modifications of the lipid composition of membranous sub-domains by lipid-modifying enzymes also contribute to vesicular traffic. In line with this, phospholipase D and calcium-independent phospholipase A2 were reported to be involved in both the transport of milk proteins

from the ER to the Golgi and in the formation of secretory vesicles from the TGN (Boisgard and Chanat, 2000; Péchoux *et al*., 2005), as was observed in other cell systems (Riebeling *et al*., 2009; Schmidt *et al*., 2010).

Trafficking steps within the secretory pathway of mammary epithelial cells and exocytosis of casein-containing vesicles with the plasma membrane might involve SNARE (soluble *N*-ethylmaleimide-sensitive fusion (NSF) attachment protein (SNAP) receptor) proteins, as already described in other cell types (Sollner *et al*., 1993; Jahn and Scheller, 2006). To date, however, only a few studies have directly addressed the functions of SNARE proteins in mammary epithelial cells. One of these suggests that VAMP-8 (vesicle-associated membrane protein 8) may be involved in casein secretion (Wang *et al*., 2007). On the other hand, SNAP-23 (synaptosomal-associated protein 23), syntaxin-3 and syntaxin-5, and Ykt6 have been described as being associated with lipid droplets (Boström *et al*., 2007; Reinhardt and Lippolis, 2008). SNAP-23 is believed to play a role in homotypic fusion of intracellular lipid droplets. Moreover, the large amount of membrane necessary for MFG secretion by budding of the APM could be partly provided by exocytosis of casein-containing vesicles (Mather and Keenan, 1998; see Fig. 14.4). Recently, the endogenous expression levels of a large set of SNAREs were investigated in mouse mammary gland (Chat *et al*., 2011). This study points to SNAP-23 as a potential central player for the coupling of casein and MFG secretion during lactation.

14.4.4 Hormonal Regulation of Milk Protein Secretion

Proteins destined for the cell exterior are secreted by either the constitutive or the regulated secretory pathway (Glombik and Gerdes, 2000; Morvan and Tooze, 2008). The fact that there is no substantial storage of newly synthesized milk proteins in mammary epithelial cells does not support the later hypothesis (Devinoy *et al*. 1995; Pauloin *et al*. 1997). On the other hand, hormones including oxytocin and prolactin are able, at least in vitro, to increase casein secretion (secretagogue effect) in mammary epithelial cells from rabbits and rodents (see Ollivier-Bousquet, 1993, 1997). Prolactin seems to act on a late step of casein trafficking, possibly exocytosis. In contrast, oxytocin was reported to also accelerate the transport of newly synthesized proteins from the ER to the Golgi apparatus and to secretory vesicles (Lollivier *et al*., 2006).

14.5 Amino Acid Transport by the Mammary Gland

Amino acids, extracted from interstitial fluid, are the major source of amino nitrogen for milk protein synthesis; therefore, a knowledge of mammary tissue amino acid transport mechanisms and their regulation is important if we are to understand fully the process of milk protein secretion. Such knowledge will help those wishing to manipulate milk protein content *via* dietary means. The uptake of amino acids across the basolateral membranes of mammary secretory cells, the major point of entry, is accomplished by an array of distinct transport mechanisms. The amino acid transporters differ from one another with respect to kinetics, substrate specificity and ion dependency; however, it is evident that they operate in a coordinated fashion to supply amino nitrogen to support milk protein synthesis.

The study of mammary epithelial amino acid transport is hampered by the relatively complex anatomy of the mammary gland. Nevertheless, the use of mammary tissue explants and the perfused mammary gland has enabled the transport of amino acids (using radiotracers) by mammary tissue to be characterized. Mammary explants are easy to prepare and have the advantage that the cellular architecture remains intact. Furthermore, the preparation of mammary explants does not require digestive agents: enzymes such as collagenase could ultimately alter the properties of membrane transport proteins. Although mammary tissue explants isolated from lactating animals are comprised of more than one cell type, it can be assumed that

the vast majority of the surface area of explants is that of the basolateral membrane of the secretory cells. Therefore, it is reasonable to assume that mammary tissue explants can be used to give a measure of amino acid transport across the blood-facing side of the mammary epithelium. The large tissue extracellular space associated with mammary explants does, however, place limitations on the design of experiments. The perfused mammary gland allows the transport of amino acids across the blood-facing aspect of the mammary epithelium to be measured under near physiological conditions: perfusates can be delivered to the gland with a flow and pressure profile similar to that found in vivo. The perfused mammary gland used in combination with a rapid, paired-tracer dilution technique allows the transport of amino acids to be measured over very short time courses (Mepham *et al*., 1985; Calvert and Shennan 1996).

14.5.1 Mammary Tissue Amino Acid Transport Systems

The identification of individual amino acid transport systems is difficult on account of the fact that a single amino acid may be able to utilize several transport systems (see Barker and Ellory 1990). Furthermore, the difficulty in characterizing amino acid transport is compounded by the lack of specific inhibitors of amino acid transporters. In spite of these drawbacks, significant progress has been made towards identifying mammary tissue amino acid transport systems together with their putative molecular correlates. Mammary tissue amino acid transport mechanisms fall into two categories: Na^+-dependent and Na^+-independent systems.

14.5.1.1 Na^+-Dependent Transport Mechanisms

Lactating mammary cells are able to concentrate free amino acids (particularly the non-essential ones) with respect to plasma, suggesting that there must be an input of free energy (Shennan *et al*., 1997). It is apparent that several amino acid transport systems utilize the electrochemical Na^+ gradient to drive the movement of amino acids into mammary cells. The Na^+-dependent mechanisms which have been identified in mammary tissue include systems A, ASC, X_{AG}^- and β.

System A prefers short-chain neutral amino acids as substrates and is characterized by its tolerance of *N*-methylated amino acids such as *N*-methylaminoisobutyrate (MeAIB). Indeed, Na^+-dependent amino acid uptake inhibited by MeAIB is usually taken as a measure of transport *via* system A. It appears that mouse, rat and bovine mammary tissue possesses system A activity (Neville *et al*., 1980; Baumrucker 1984; Verma and Kansal 1993; Shennan and McNeillie 1994a). Lopez *et al.* (2006) provided convincing evidence that SNAT2 may be the molecular correlate of system A in rat mammary tissue. In contrast, no evidence for the presence of system A at the blood-facing aspect of the guinea pig mammary gland could be found (Mepham *et al*., 1985). The limited kinetic data available suggest that system A in lactating mammary tissue operates with relatively low affinity: the K_M of methionine and α-aminoisobutyric acid uptake via system A in mouse mammary tissue is 0.47 mM and 2.0 mM, respectively (Neville *et al*., 1980; Verma and Kansal, 1993). Several lines of evidence suggest that system A is regulated by milk stasis, by starvation and by the stage of lactation (Neville *et al*., 1980; Shennan and McNeillie 1994c; Verma and Kansal 1995). In accordance with these findings, SNAT2 mRNA expression, respectively, increases and decreases during lactation and weaning. Moreover, the abundance of SNAT2 mRNA is increased by oestrogen which may explain the high levels observed during pregnancy (Lopez *et al*., 2006). System A activity is regulated by prolactin: treating animals with bromocriptine, a drug which inhibits prolactin secretion from the pituitary gland, markedly reduces the arteriovenous concentration differences of amino acids which are potential substrates of the A system (Vina *et al*., 1981).

Mammary tissue from several species (e.g., bovine, mouse, guinea pig) has been shown to posses system ASC (Baumrucker 1985; Mepham *et al*., 1985; Verma and Kansal 1993). It has been established in other tissues that this mechanism

cotransports neutral amino acids such as alanine, threonine and cysteine with Na^+. System ASC in mammary tissue, like system A, operates with relatively low affinity: the K_M of methionine uptake by lactating mouse mammary tissue via system ASC is 0.46 mM (Verma and Kansal, 1993). At present, there appears to be a paucity of information on the regulation of mammary tissue amino acid transport via system ASC except for the finding that starvation gives rise to a large increase in system ASC activity in mouse mammary tissue (Verma and Kansal, 1995). There are at least two molecular isoforms of system ASC, both of which have been detected in mammary tissue. Thus, the expression of ASCT1 and ASCT2 mRNA has respectively been described in lactating rat and porcine mammary tissue (Aleman *et al*., 2009; Laspiur *et al*., 2009). The expression of both transcripts increases with the onset of lactation.

The transport of anionic amino acids by lactating mammary tissue has been extensively studied (Millar *et al*., 1996, 1997a, b). The predominant, if not the only, pathway for L-glutamate and L-aspartate transport is a high-affinity ($K_M = 18$ μM for L-glutamate), Na^+-dependent system analogous to system X_{AG}^- (Kanai *et al*., 1994). The Na^+-dependent anionic amino acid carrier is very selective for anionic amino acids: it does not interact with neutral or cationic amino acids (Millar *et al*., 1996, 1997b). An unusual feature of system X_{AG}^- is the ability to discriminate between the opical isomers of glutamate but not those of aspartate. The high-affinity anionic amino acid carrier is able to act as an exchange system as well as a cotransport mechanism suggesting that the transport of L-glutamate will affect the intracellular concentration of L-aspartate (and vice versa) (Millar *et al*., 1997b). Several high-affinity anionic amino acid carriers, which have identity with system X-AG, have been cloned and characterized (e.g., see Kanai and Hediger, 1992; Pines *et al*., 1992; Storck *et al*., 1992). Three clones, EAAC1, GLAST and GLT-1, have been identified in rat mammary tissue (Martinez-Lopez *et al*., 1999; Aleman *et al*., 2009). However, the exact contribution of these isoforms to anionic amino acid transport across the basolateral membranes of mammary secretory cells is unknown.

Taurine, a nonprotein amino acid, is taken up by lactating rat and porcine mammary tissue *via* a high-affinity, Na^+-dependent transport system analogous to system β (Shennan and McNeillie, 1994b; Bryson *et al.,* 2001). The mammary (Na^++taurine) cotransport system also requires Cl^- for maximal activity. System β has narrow substrate specificity: only β-amino acids such as taurine and β-alanine interact with the transporter. The activity of the rat mammary (Na^+-Cl^--taurine) cotransporter decreases as lactation progresses (Millar and Shennan 1999). In accordance with this, Aleman *et al*. (2009) have reported that the expression of rB16 (a cloned taurine transporter) mRNA decreases between early and peak lactation. The lactating gerbil mammary gland expresses a high-affinity (Na^+-taurine) cotransport system which, unlike the rat mammary taurine transporter, is not dependent upon Cl^- (Shennan, 1995).

14.5.1.2 Na^+-Independent Transport Mechanisms

System L has been identified in mouse, rat, guinea pig and bovine mammary tissue (Neville *et al*., 1980; Mepham *et al*., 1985; Verma and Kansal, 1993; Shennan and McNeillie, 1994c). System L is a Na^+-independent transport mechanism that has wide substrate specificity. Indeed, system L may be the most important transport system for the uptake of essential neutral amino acids by the lactating mammary gland. Na^+-independent amino acid transport sensitive to 2-aminobicycloheptane-2-carboxylic acid (BCH) is taken as a measure of system L activity. It is generally accepted that system L can act as an amino acid exchange mechanism. Accordingly, methionine uptake by mouse mammary gland *via* system L can be *trans*-accelerated by intracellular amino acids. However, amino acid efflux from rat mammary tissue, *via* system L, is not *trans*-stimulated by extracellular amino acids, suggesting that the transporter operates with asymmetric kinetics which could favour the retention of substrates within the gland. System L has been localized to the basolateral aspect of the lactating rat mammary gland (Shennan *et al*., 2002). There are at least four molecular isoforms of system L

(LAT 1–4), two of which have been described in the mammary gland. Thus, LAT1 and LAT2 mRNA are expressed in the rat mammary gland (Shennan *et al*., 2002; Aleman *et al*., 2009). Interestingly, Aleman *et al*. (2009) have shown that LAT1 mRNA expression in rat mammary tissue markedly increases during lactation. LAT mRNA is also expressed in the mouse and bovine mammary gland (Rudolph *et al*., 2007; Finucane *et al*., 2008; Connor *et al*., 2008). The expression of LAT1 mRNA increases during the transition from pregnancy to lactation and is also up-regulated by milking frequency.

The transport of cationic amino acids by lactating mammary tissue is a Na^+-independent process (Baumrucker 1984; Shennan *et al*., 1994b; Hurley *et al*., 2000). The pathway for lysine and arginine uptake by bovine mammary tissue is not affected by replacing extracellular Na^+ and appears to be relatively specific for cationic amino acids. On this basis Baumrucker (1984) concluded that cationic amino acids are transported via system y^+. There is evidence suggesting that CAT-1 may be responsible for system y^+ activity in rat mammary tissue (Aleman *et al*., 2009). CAT-1 mRNA expression is low in mammary tissue isolated from pregnant rats and increases with the onset of lactation (Aleman *et al*., 2009). Transcripts for two cloned Na^+-independent cationic amino acid transporters, CAT-1 and CAT-2B, have been detected in lactating porcine tissue (Laspiur *et al*., 2009), However, it appears that the expression of both transcripts is relatively unaffected by the stage of lactation. Cationic amino acid uptake by lactating rat mammary tissue is also facilitated by a transporter which interacts with neutral amino acids such as glutamine and leucine (Shennan *et al*., 1994a; Calvert and Shennan 1996). Thus, certain neutral amino acids respectively inhibit and stimulate lysine uptake by and efflux from rat mammary tissue. It is likely that this pathway is y^+L: y+LAT1 mRNA has been detected in lactating rat mammary tissue (Boyd, Kudo and Shennan, unpublished).

System T, a mechanism specific for aromatic amino acids, has been described in lactating mouse mammary tissue (Kansal and Kansal, 1996). Thus, the moiety of tyrosine uptake by mouse mammary tissue which is not dependent upon extracellular Na^+ and is not sensitive to BCH has been ascribed to system T. This mechanism operates with low affinity; the K_M for tyrosine transport is approximately 15 mM.

14.5.1.3 Volume-Activated Amino Acid Transport

To survive, cells have to regulate their volume within relatively narrow limits (see Hoffmann and Simonsen, 1989, for a review). Cell membranes are very permeable to water which means that cell volume, otherwise termed the cellular hydration state, will be determined by the osmolarity of the extracellular fluid and by the intracellular content of osmotically active solutes. The cellular hydration state can be changed as a consequence of anisosmotic conditions, cellular accumulation of solutes or oxidative metabolism. Cells are able to regulate their volume following swelling or shrinking. Cell volume regulation involves the transmembrane movement of solutes together with osmotically obliged water (Hoffmann and Simonsen, 1989).

A knowledge of volume-regulatory processes in mammary tissue is of particular importance given that cell volume changes markedly affect the rate of milk protein synthesis (Millar *et al*., 1997a). It has been established that cell swelling activates the transport of amino acids in mammary tissue (Shennan *et al*., 1994; 1996). Thus, cell swelling, induced by a hypoosmotic shock, increases the efflux of amino acids such as taurine and glycine via a pathway which has the characteristics of a channel rather than a carrier. The swelling-induced amino acid efflux pathway appears to be situated in the blood-facing aspect of the mammary epithelium (Calvert and Shennan, 1998). The volume-activated efflux of taurine is dependent upon the extent of cell swelling and can be inhibited by a number of pharmacological agents such as niflumic acid (Shennan *et al*., 1996). There is the strong possibility that volume-activated amino acid efflux may participate in mammary cell volume regulation given that amino acids are concentrated within mammary cells with respect to plasma.

14.5.2 Transport and Metabolism of Peptides

Although free amino acids are the major source of amino nitrogen for milk protein synthesis, it is evident that the uptake of several essential amino acids is less than their output in milk, suggesting that other circulating forms of amino acids, such as peptides, may be available for casein production (e.g., see Backwell *et al*., 1996). In this connection, it has been demonstrated that the goat mammary gland is able to use intravenously administered peptides for milk protein synthesis (Backwell *et al*., 1994, 1996). The in vivo studies were unable to show whether the mammary gland transported peptides intact or whether the peptides were hydrolysed extracellularly followed by uptake of the liberated amino acids. It has been shown, albeit indirectly, that the mouse mammary tissue does not significantly hydrolyse peptides extracellularly but is able to transport peptides intact (Wang *et al*., 1996). On the other hand, studies using the rat mammary gland as a model suggest that mammary tissue is able to both transport and hydrolyse dipeptides (Shennan *et al*., 1998, 1999). It is evident that the perfused lactating rat mammary is able to transport dipeptides which are resistant to hydrolysis (i.e., D-Phe-L-Gln; D-Phe-L-Glu); the nature of the pathway remains to be identified precisely. However, it is apparent that the capacity of the rat mammary gland to transport peptides intact across the basolateral pole of the epithelium is relatively limited (Shennan *et al*., 1998). Transcripts for two proton-dependent peptide transporters, namely, PepT1 and PepT2, have been identified in lactating rat and human mammary epithelial cells (Alcorn *et al*., 2002; Groneberg *et al*., 2002; Gilchrist and Alcorn, 2010). PepT2 protein is localized in the apical membrane of rat ductal epithelial cells and is therefore not in a position to facilitate the uptake of peptides from the circulation. Rather, it is likely that PepT2 provides a route for the reuptake of peptides from milk as postulated by Shennan and Peaker (2000).

Rat mammary tissue is able to extensively hydrolyse peptides extracellularly: this has been demonstrated using both direct and indirect experimental approaches (Shennan *et al*., 1998; 1999). Anionic dipeptides presented to rat mammary tissue can *trans*-stimulate D-aspartate efflux *via* the high-affinity anionic amino acid carrier. If it is accepted that dipeptides do not interact directly with the amino acid carrier, then it can be assumed that anionic amino acids, produced as a consequence of extracellular hydrolysis, act to stimulate D-aspartate efflux. In this connection, it has been shown that mammary tissue is capable of hydrolysing a variety of aminoacyl-*p*-nitroanilides (peptide analogues). Quantitatively, it appears, at least in the rat, that hydrolysis of peptides followed by uptake of the free amino acids may be more important than the transport of peptides (Shennan *et al*., 1998).

The identity of the peptidases involved in the extracellular hydrolysis of peptides by mammary tissue has not been established. However, γ-glutamyltranspeptidase appears to be involved given that glutathione can stimulate D-aspartate efflux from rat mammary tissue in a fashion sensitive to acivicin (Shennan *et al*., 1998). The hydrolysis of glutathione may be an important route for providing the mammary gland with cysteine. There is evidence to suggest that aminopeptidase N plays a role in providing free amino acids for protein synthesis in the goat mammary gland (Liu *et al*., 2010).

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Genetic Polymorphism of Milk Proteins

15

P. Martin, L. Bianchi, C. Cebo, and G. Miranda

15.1 Introduction

Since the discovery, over half a century ago, of two electrophoretically distinct forms of β-lactoglobulin by Aschaffenburg and Drewry (1957), genetic polymorphism of milk proteins has been extensively investigated in cattle. For obvious economic reasons most of the efforts have been focused on the impact on milk processing (particularly cheesemaking properties). However, genetic polymorphisms of milk proteins have been also used to analyse, through association studies, possible relationships with quantitative and qualitative milk traits as well as evolutionary and biodiversity issues. Most of these aspects have been addressed in depth in the previous versions of the chapter in this series dedicated to genetic polymorphism of milk proteins by Ng-Kwai-Hang and Grosclaude (1992, 2003).

Since Caroli *et al.* (2009) recently reviewed milk protein polymorphisms in cattle, focusing mainly on the effect on animal breeding and human nutrition, species other than bovine will be particularly considered here and the main focus of this account will be on advances made since the previous chapter in this series (Ng-Kwai-Hang and Grosclaude, 2003) on the molecular bases for genetic polymorphism at the genome level.

With the advent of molecular biology and DNA analysis, search for polymorphism progressively moved from qualitative to quantitative aspects (from protein to genome), and with the availability of next-generation sequencing (NGS) technologies that have revolutionised genomics and genetics, this trend has been accentuated to focus on a large number of outstanding issues that previously could not be addressed effectively. Thus, we are now able to study genetic variation on a genome-wide scale and characterise gene regulatory processes at unprecedented resolution (Gilad *et al.*, 2009).

One of the main goals of genomics is to determine the genetic differences among individuals and to understand their relationships to the phenotypic differences within species. Structural variations within the genome have been described in a number of species. These variations consist in single-nucleotide polymorphisms (SNPs) and structural variations (SVs) including short insertions/deletions (indels) and other more complex ones such as duplications and translocations. SNPs and SVs have been shown to account for ca. 83% and 17%, respectively, of the total detected genetic variation in gene expression (Stranger *et al.*, 2007). In humans, SVs have been associated with complex human traits, such as autism, schizophrenia,

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Crohn's disease and susceptibility to HIV infection (Zhang *et al.*, 2009). For most other species, including the major farm animals, the extent and biological consequences of SVs remain rather poorly documented (Kerstens *et al.*, 2011). Because of the efficiency of genotyping methods and the central role they play in the genome-wide association studies, SNPs are currently the best known and useful genetic variations. However, SVs which have been much less studied due to the lack of a cost-effective approach for genotyping start to be considered and their genetic significance recognised (Zhang *et al.*, 2011), but the effect that SVs have on gene expression is likely underestimated given the much less completeness and accuracy with which SVs could be queried.

Most of the studies reporting genetic polymorphisms of milk proteins are related to the colloidal and soluble fraction of milk, namely, caseins and whey proteins. However, growing attention is paid to milk-fat globule membrane (MFGM)-associated proteins. Although these proteins account for only 1–2% of total milk proteins, evidence that MFGM proteins possess techno-functional and nutritional properties is accumulating (Dewettinck *et al.*, 2008). Since data on the genetic polymorphism of these proteins is becoming more consistent, a part of this chapter will be devoted to this issue.

15.2 Methods of Detecting Genetic Polymorphism

Methods of detecting genetic polymorphism have been discussed in detail in the previous version of this chapter. Briefly, genetic polymorphisms can be detected at the phenotypic level using classical techniques to analyse proteins such as electrophoresis, isoelectric focusing (IEF) or chromatography. These techniques detect only genetic variations resulting in differences on the net charge, hydrophobicity or molecular weight of the protein. For the phenotyping of breeds and large populations, IEF is still the most effective and convenient method since it gives simultaneously an overview of the phenotype expression of the six main milk protein genes. However, techniques using mass spectrometry, which has increasing resolution and is fast and efficient, are beginning to supplant IEF.

Our first purpose here is to introduce recent developments in mass spectrometry coupled with separation techniques to analyse milk protein polymorphisms which are the most powerful methods available nowadays to detect and analyse genetic polymorphisms at the DNA level.

15.2.1 Recent Developments in Phenotyping Methods

The profiling of milk proteins is complicated by the existence of genetic polymorphisms, alternative splicing variants and post-translational modifications (PTMs). As a result, several forms of a same protein family are usually present together in a given milk sample, rendering the analysis of the lacto-proteome a particularly complex matter. Over the years, several electrophoretic and chromatographic techniques have been applied (reviewed in detail by Ng-Kwai-Hang and Grosclaude, 2003) to the routine screening of individual milk samples. Electrophoresis and, to a lesser extent, chromatography have made it possible to detect the vast majority of the currently known milk protein variants. Over the last 15 years, however, techniques based on the mass spectrometry analysis of proteins and peptides have started to be applied to milk proteins. Mass spectrometry analyses offer the undoubted advantage of detecting minimal differences in terms of molecular mass, even among residues having similar chemical properties. Thus, the possibility to detect new polymorphisms is greatly increased, in that theoretically all amino acid modifications imply a molecular mass shift in the mature protein, with the sole possible exception of isobaric amino acids, such as leucine/isoleucine (that remain undetectable using the current techniques) or lysine/glutamine.

Remarkable progress has been made in top-down (TD) MS that has gained a remarkable space in proteomics, rapidly trespassing the classical bottom-up approaches and surpassing the limit between a promising approach and a solid established technique (Armirotti and Damonte, 2010). Using the so-called "top-down" process,

in which proteins are analysed in the gas phase as intact molecules, it is possible to derive structural information on proteins with the level of accuracy that is impossible to achieve by classical bottom-up approaches. Complete maps of PTMs and assessment of single amino acid polymorphisms are only a few of the results that can be obtained with this technique. Despite some existing technical and economic limitations, TD analysis is at present the most powerful instrument for MS-based proteomics. Such TD approaches have been used to analyse phosphoproteins, including caseins (Wu *et al*., 2009), and *O*-glycosylation of MUC-1 and κ-casein (Hanisch, 2011).

Beforehand, however, classical mass spectrometric approaches had been first applied in the detection of a milk protein variant by Visser *et al*. (1995), and, then by Dong and Ng-Kwai-Hang (1998) and Senocq *et al*. (2002) who characterised variants G (P152L), F (P137L) and H (M93L) of bovine β-casein, respectively. In particular, the peptides carrying the mutation in variants G and H were sequenced by tandem mass spectrometry (MS/MS). Ferranti *et al*. (2001) and Pierre *et al.* (2001) proposed the use of ESI-MS for the routine screening of ovine and caprine caseomes, respectively. They were able to detect genetic variants, together with their non-allelic forms, of α_{s1}- and α_{s2}-caseins, and different phosphorylation levels. Neveu *et al.* (2002) reported the existence of a new variant of caprine β-casein by LC-MS, and the phosphorylation patterns of the protein were characterised by the combined use of peptide mass fingerprinting and sequencing by tandem mass spectrometry. The existence of a truncated form of caprine β-casein associated with the O′ allele and its amino acid sequence was proposed by Cunsolo *et al*. (2005). The same research group described two truncated forms (204 instead of 207 AA) of caprine α_{s2}-casein and speculated that these proteins could be the product of a differential splicing of pre-messenger RNA encoding α_{s2}-casein alleles A and E during their maturation process (Cunsolo *et al*., 2006). LC-MS/MS and MALDI-PSD-TOF-MS were applied, respectively, for the characterisation of ovine α_{s1}- (Chianese *et al.*, 1997a, b) and α_{s2}-casein (Picariello *et al*., 2009a) variants (see below).

Mass spectrometric techniques have therefore proved to be useful tools for the screening of milk protein polymorphisms. On this basis, the more recent trends aim at developing quantitative or semi-quantitative tools to assess the presence of given casein variants in bulk milk from the same species (Picariello *et al*., 2009b) or the fraudulent presence of milk from other species, as adulteration of milk for the dairy industry (Nicolaou *et al.*, 2011; Cuollo *et al.*, 2010).

15.2.2 Methods of Detecting Genetic Polymorphisms at the DNA Level

Analyses of milk protein genes at the nucleotide level started 25 years ago with cDNA cloning and Sanger's method for DNA sequencing, providing us with the most basic information of all: the sequence of nucleotides. Few years later, with the advent of the polymerase chain reaction (PCR) technique (Saiki *et al.*, 1988), amplification of specific gene regions (including exons) at the genomic level has facilitated the analysis of polymorphisms within the coding sequences and provided tools to genotype animals, including males and females prior to lactation.

Capillary sequencing is no longer the technology of choice for most ultra-high-throughput applications. We are now witnessing a genomic revolution due to the continued advancements in the next-generation sequencing technologies which assist solving complex biological problems. Genome sequences are now available for a number of domestic species, including mammals, and with them high-throughput tools including high-density single-nucleotide polymorphism (SNP) panels. As a result, domestic animal populations are becoming invaluable resources for studying the molecular architecture of complex traits. Recent progress in the positional identification of genes underlying complex traits in domestic animals has been reviewed by Georges (2007), and the importance of comparative genomics for dissecting the genetic basis of phenotypic variation has been stressed

(Andersson and Georges, 2004). Obviously, this massive sequencing at the nucleotide level highlights the occurrence of new genetic polymorphism of which molecular bases are easily identified. This situation differs from our current concept of genetic polymorphism which moves from qualitative variability (protein variants) to a notion integrating a quantitative variability dimension. Thus, emphasis is placed on new polymorphisms impacting not only milk protein structure but also its expression. Polymorphisms occurring in *cis*-regulatory elements (mainly within the 5′-flanking region of transcription units encoding milk proteins) have been reported, as well as insertion/deletion (indel) and SNP within exon, intron and/or 3′-untranslated sequences. Mutations responsible for the occurrence of premature stop codons have been shown to be associated both with a decrease in the level of the relevant transcripts and the existence of multiple forms of messengers due to alternative splicing (exon skipping, usage of cryptic splice sites). Such a situation, well exemplified by the gene encoding α_{s1}-casein in the goat, may have dramatic biological consequences (secretion pathway, casein micelle structure, fat content, etc.) by modifying the message and accordingly the primary structure of the protein as well as its expression (Martin *et al*., 2002).

Rather than to make an exhaustive review of the abundant literature existing in this field, we have chosen to focus on some demonstrative examples, such as β-lactoglobulin in cattle and α_{s1}- and β-caseins in goats, to show how mutations responsible for polymorphisms at the genomic level can influence milk protein composition, both at the qualitative and quantitative levels. Beforehand, we will update the state of our current knowledge of the molecular bases for genetic polymorphism of milk proteins.

15.3 Molecular Basis for Genetic Polymorphism of Milk Proteins

A genetic polymorphism is due to the occurrence of different alleles at the same locus, which may code for different polypeptide chains (protein variants), or not. Several alleles can actually give rise to a same variant (*silent alleles*), namely, when a nucleotide substitution does not modify the coding message (*synonymous mutation*) or when mutations occur in a portion of the gene excluded from the mature transcript during the course of the splicing process (*introns*) or located within untranslated regions of the messenger or even when the sequence of the signal peptide which is removed during the secretion process to produce the mature protein is modified.

Most protein variants originate from single-nucleotide mutations in the coding sequence of the parent DNA, thus leading to amino acid substitutions (*non-synonymous mutation*). The resulting amino acid, in turn, can modify the physico-chemical properties (net charge, isoelectric point, phosphorylation or hydrophobicity) of the protein. Substitution of a single nucleotide occurring in intron consensus splice sequences can also alter the maturation process of messengers, and as a result, one or several exons can be skipped in the mature mRNA. Deletion of a single nucleotide in coding exon sequences usually causes the occurrence of a premature stop codon interrupting the reading frame and promoting nonsense-mediated mRNA decay. In turn, these events result in the appearance of internally deleted (exon skipping) or virtually truncated forms of the mature protein. This is frequently associated with a strong reduction or even the absence of protein synthesis due to degradation of the artefactual transcripts (see below). Deleted forms have been described, so far in most species, in the three calcium-sensitive caseins (α_{s1}-, α_{s2}- and β-caseins), whereas a truncated form has been to date reported and characterised only for the goat β-casein (Cunsolo *et al*., 2005). To our knowledge, the κ-casein gene has never been reported to contain exon-skipping events. This is likely due to functional constraints. The gene consists of five exons, of which only three are coding (exons 2, 3 and 4), the major part of the protein being encoded by exon 4 and the signal peptide by both exons 2 and 3. An exception to the general rule described above is the goat α_{s1}-casein variant M, detected in the Italian Montefalcone breed which was suggested to

originate from an interallelic recombination event between two phylogenetically distinct parent alleles (Bevilacqua *et al.*, 2002).

In their study on the so-called, at the time, "α_s-casein" fraction in goat milk, Richardson and Creamer (1975) did not detect a fraction corresponding to α_{s1}-casein. These conclusions were later corrected by Boulanger *et al.* (1984) who not only detected and sequenced the protein but were able to describe at least three variants, named A, B and C. They stated that the apparent lack of α_{s1}-casein in some samples was in fact due to a genetically controlled strongly reduced rate of expression. It was the first example of genetic polymorphism associated with a quantitative variability (*quantitative polymorphism*), reported regarding milk proteins.

Goat α_{s1}-casein is a paradigm of this kind of polymorphism. Indeed, alleles associated with at least four levels of synthesis have been described so far, with the actual concentration in milk being the arithmetic sum of the contribution of each allele in homozygous and heterozygous subjects (Grosclaude and Martin, 1997). Depending on their level of expression, alleles are therefore referred to "strong" (3.6 g/L per allele), "intermediate" (1.6 g/L), "weak" (0.6 g/L) and "null" (non-detectable amounts) alleles. At least one "null" allele has been described in the goat species for the three calcium-sensitive caseins (Leroux *et al.*, 1990; Mahé and Grosclaude, 1993; Persuy *et al.*, 1999; Ramunno *et al.*, 2001a, 2005; Cosenza *et al.*, 2003; Ådnøy *et al.*, 2003) but, interestingly, not for κ-casein, not surprisingly however given its stabilising function of casein micelles in milk. Quantitative polymorphisms were also described for bovine α_{s1}- and κ-caseins (Rando *et al.*, 1998; Damiani *et al.*, 2000) and β-lactoglobulin (BLG) in various breeds (reviewed by Braunschweig and Leeb, 2006), but none was reported so far in the ovine species, except a moderate reduction in α_{s1}-casein secretion associated with variant H (Giambra *et al.*, 2010a, b).

General mechanisms controlling gene expression act both at the transcriptional and post-transcriptional levels. Multiple specific factors exert control of transcription: the strength of promoter elements, the presence or absence of enhancer sequences and the interaction between multiple activator proteins and inhibitor proteins. Polymorphisms present in *cis*-regulatory elements, mainly within the 5′-flanking region of transcription units encoding milk proteins, have been found (reviewed by Martin *et al.*, 2002). Our current understanding of regulatory polymorphisms of milk protein genes is growing. Mechanisms are complex and regulation of milk protein expression is mostly controlled by the non-coding portion of the genome, through a series of complex mechanisms acting at several levels, including pre-mRNA splicing (as already mentioned) and export, mRNA stability and translation (Bevilacqua *et al.*, 2006).

Unstable transcripts have sequences (predominately, but not exclusively, in the 3′-untranslated regions) that are signals for rapid degradation. Insertions of repetitive sequences, such as relicts of long interspersed elements (LINE), have been also described to influence mRNA stability (Jansà-Perez *et al.*, 1994). In addition, in the very recent years, a new model of gene regulation has emerged that involves control exerted by small non-coding RNAs. This small RNA-mediated control can be exerted either at the level of the translatability of the messengers or their stability. A nice example has been provided by Liao *et al.* (2010), who showed that lactoferrin gene expression and function are directly targeted by miR-214 in HC11 and MCF7 cells. In the lactoferrin mRNA 3′-untranslated region of human, mouse, rat, pig, bovine, camel and goat species, there is a conserved region that perfectly matches the seed region of miR-214.

15.4 Genetic Polymorphism of Milk Proteins in Dairy Ruminants

The aim of this section is to present the current knowledge in terms of genetic polymorphism for the major milk proteins of dairy ruminants, namely, cow, goats and sheep. It will deal with the four caseins (α_{s1}, β, α_{s2} and κ) and the major whey proteins (BLG and α-lactalbumin), and only mutations leading to a well-characterised qualitative or quantitative protein polymorphism will be considered. The multiple silent alleles coding for a same protein variant will therefore not be described.

Since the last edition of this book, genetic polymorphism of bovine milk proteins was the subject of two comprehensive reviews (Farrell *et al*., 2004; Caroli *et al*., 2009), in which the current nomenclature and recent findings were given. There have also been two reviews by Marletta *et al*. (2007) and Amigo *et al*. (2000) dealing with goat caseins and the ovine major milk proteins, respectively. Over the last decade, however, nucleic acid-based approaches which represent the highest throughput and best overall methods for obtaining information at the genome level as well as proteomics-based approaches relying on mass spectrometry methods for the detection and characterisation of milk protein polymorphisms have led to significant advances on the subject, mainly concerning small ruminants and, to a lesser extent, cattle.

Where possible, the description of a given protein variant was integrated in tables with amino acid modification, GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and UniProt (http://www.uniprot.org/) accession numbers, as well as with relevant bibliographic references. Nevertheless, such information was not always available. Often, variants were detected and confirmed by classical electrophoresis techniques, but the primary structure and the allele sequence were not characterised. Likewise, databases or reviews reported sequence conflicts or misinterpretations that the present chapter aims at resolving.

Lastly, according to the current nomenclature, the variants ascribed to bovine, caprine and ovine milk do include sequences found, respectively, in the genera *Bos*, *Capra* and *Ovis* and not only in domesticated cattle, goat and sheep.

15.4.1 Bovine Milk Proteins

15.4.1.1 Bovine α_{s1}-Casein

Bovine α_{s1}-casein was first sequenced at the protein level by Mercier *et al*. (1971) and Grosclaude *et al*. (1973) and then at the cDNA level by Nagao *et al*. (1984) and Stewart *et al*. (1984), who were thus able to establish the sequence of the signal peptide. The mature protein is a 199-residue polypeptide chain, carrying ten potential phosphorylation sites (9 serine and 1 threonine), of which seven are clustered at positions 46–49 (SerP-Glu-SerP-ThrP) and 64–68 (SerP-Ile-SerP-SerP-SerP). The bovine protein, unlike the mouse α_s-casein, contains no cysteine residues. Nine genetic variants have been characterised to date and are presented in Table 15.1. Protein variants differ mainly by single amino acid substitutions, with the exceptions of A (Grosclaude *et al*., 1972) and H (Mahé *et al*., 1999), which are internally deleted forms lacking, respectively, sequences encoded by exon 4 and exon 8. Variant G that shares the same primary structure as variant B is characterised by a reduction of the α_{s1}-casein content in milk, reaching ca. 55% (Mariani *et al*., 1995; Rando *et al*., 1998). Moreover, the single amino acid substitution of a potentially phosphorylated serine to a leucine, at position 66 in variant F (Prinzenberg *et al*., 1998), results in the loss of a further potential phosphorylation site at position 64. It was postulated that variant I is caused by a non-synonymous nucleotide substitution in exon 11 of the gene and that it originated within *Bos indicus* and spread subsequently to *Bos taurus* (Lühken *et al.,* 2009).

15.4.1.2 Bovine β-Casein

The sequence of bovine β-casein was first established at the protein level by Ribadeau Dumas *et al.* (1972). Carles *et al*. (1988), using a new strategy for primary structure determination of proteins, reported a sequence which currently refers to variant A^1. The A^2 variant sequence was determined by cDNA sequencing (Stewart *et al*., 1987). The protein is secreted as a 209-residue peptide chain, containing no cysteine and with six potential phosphorylation sites (5 serine and 1 threonine), of which four are clustered at positions 15–19 (SerP-Leu-SerP-SerP-SerP).

Compared to previous reviews by Farrell *et al*. (2004) and Caroli *et al.* (2009), the number of variants (12) remains unchanged, although some modifications are presented (Table 15.2). The variant previously named H1 (Han *et al*., 2000) was excluded, since the mutation leading to the substitution R25C (Arg/Cys at position 25, previous GenBank accessions AF104928 and AF104929) was not confirmed by re-sequencing (present GeneBank accession AH007287). The variant previously named H2, described by Senocq *et al*. (2002), is therefore renamed H. More recently, Miranda *et al.* (submitted) have

Table 15.1 Changes in bovine α_{s1}-casein variants (alleles)

	Position										
Variant/allele	14–26	53	51–58	59	64	66	84	192	GenBank accession	SwissProt accession	References
A	DEL										Grosclaude *et al*. (1972)
B		**Ala**		**Gln**	**SerP**	**SerP**	**Glu**	**Glu**	X59856	**P02662**	Mercier *et al*. (1971) and Grosclaude *et al*. (1973)
C								Gly			Grosclaude *et al*. (1972)
D		ThrP									Grosclaude *et al*. (1972)
E				Lys				Gly			Grosclaude *et al*. (1972)
F					Ser	Leu					Prinzenberg *et al*. (1998)
G											Rando *et al*. (1998)
H			DEL								Mahé *et al*. (1999)
I							Asp	Gly	U862370/371 (*Bos indicus*) EU908730 (*Bos taurus*)		Lühken *et al*. (2009, Balteanu *et al*. (2008, 2010)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column
DEL: internal deletion of the corresponding sequence in the mature protein

Table 15.2 Changes in bovine β-casein variants (alleles)

Variant/allele	Position 18	35	36	37	52	67	72	93	106	122	138	152	? (114–169)	GenBank accession	SwissProt accession	References
A1						His								X14711		Peterson and Kopfler (1966) and Carles *et al.* (1988)
A2	**SerP**	**SerP**	**Glu**	**Glu**	**Phe**	**Pro**	**Gln**	**Met**	**His**	**Ser**	**Pro**	**Pro**	**Gln**	**M16645**	**P02666**	Peterson and Kopfler (1966), Yan and Wold (1984) and Stewart *et al.* (1987)
A3									Gln					NM_181008		Peterson and Kopfler (1966)
B						His				Arg				BC111172		Aschaffenburg (1961)
C		Ser		Lys		His										Aschaffenburg (1961)
D	Lys															Aschaffenburg *et al.* (1968)
E			Lys													Voglino (1972)
F						His						Leu				Visser *et al.* (1995)
G						His					Leu[a]					Dong and Ng-Kwai-Hang (1998)
H							Glu	Leu					Glu[b]			Senocq *et al.* (2002)
I								Leu						AY366419		Jann *et al.* (2002)
J					Ser											Miranda *et al.* (submitted)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column
[a]Originally identified as P137L by Dong and Ng-Kwai-Hang (1998), the amino acid modification has been assigned to position 138 according to the given reference sequence
[b]The authors located a Gln to Glu substitution in the 114–169 sequence of the mature protein but could not establish more precisely its position

Table 15.3 Changes in bovine α_{s2}-casein variants (alleles)

	Position					GenBank	SwissProt	
Variant/allele	8	33	47	51–59	130	accession	accession	References
A	**SerP**	**Glu**	**Ala**		**Thr**	**M16644** **M94327**	**P02663**	Stewart *et al*. (1987) and Groenen *et al*. (1993)
B	Phe							Ibeagha-Awemu *et al*. (2007)
C		Gly	Thr		Ile			Mahé and Grosclaude (1982)
D				DEL				Bouniol *et al*. (1993a, b)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column
DEL: internal deletion of the corresponding sequence in the mature protein

detected and characterised a novel variant (named J), both at the protein and at the genomic DNA level, differing from A2 for a single amino acid substitution (phenylalanine to serine at position 52) which does not result in a new potential phosphorylation site.

15.4.1.3 Bovine α_{s2}-Casein

The primary structure of bovine α_{s2}-casein was obtained by amino acid sequencing (Brignon *et al*., 1977) and subsequently corrected by cDNA sequencing (Stewart *et al*., 1987). The gene sequence is also available (GeneBank accession M94327). The mature protein is a polypeptide of 207 amino acids, displaying 17 potential phosphorylation sites (12 on serine and five on threonine residues), nine of which are clustered within three short segments of the molecule: 8SerP-SerP-SerP10, 56SerP-SerP-SerP58 and 129SerP-ThrP-Ser131. Several α_{s2}-casein isoforms are present in bovine milk, differing in the level of phosphorylation (10–13 phosphate groups/molecule). Bovine α_{s2}-casein contains two cysteine residues involved in intra-chain disulphide bridges in monomers of α_{s2}-casein isolated from milk (Rasmussen *et al*., 1994).

Four genetic variants have been described, named A to D, as shown in Table 15.3, and no major modifications have been reported since the review by Caroli *et al*. (2009), except the Ser/Phe substitution observed at position 8 (S8F) in variant B, resulting in the loss of a potential phosphorylation site (Ibeagha-Awemu *et al*., 2007). Variant C differs at three positions from variant A (Mahé and Grosclaude, 1982). Lastly, the internal deletion of eight residues encoded by exon 8 (Bouniol *et al*., 1993a, b), results in the loss of an entire cluster of phosphorylated serines.

15.4.1.4 Bovine κ-Casein

The primary structure of bovine κ-casein and of its signal peptide was predicted by cDNA sequencing (Stewart *et al*., 1984). The mature protein has 169 amino acid residues, with two cysteines and six potential phosphorylation sites (two serine and four threonine), and up to six glycosylation sites have been described (Pisano *et al*., 1994). The variants so far described for κ-casein are shown in Table 15.4. No modifications are proposed to the review article by Caroli *et al*. (2009), whereas the amino acid substitutions ascribed to variants F2 and G1 by Farrell *et al*. (2004) are inconsistent with those published by the original authors. Compared to the previous reports, two additional protein variants were included, here named K and L, both predicted from sequencing of exon 4 in domesticated yak samples. Variant K (GenBank accession number AF194989) carries the substitution already observed in G2 (D148A) with two additional amino acid substitutions (P36L and P130R). Variant L has the same D148A mutation, which is common to all the yak sequences so far, but carries in addition a unique insertion of four amino acids, corresponding to the duplication of sequence 148–151 (ASPE) found in bovine allele B. Therefore, the predicted primary structure of the mature protein contains 173 amino acid residues (Prinzenberg *et al*., 2008). The description of variants will not be discussed in detail, but attention can be drawn on mutations involving R10 (variant F2) and R97 (variants C, D and G1) and P130, resulting either in the loss

Table 15.4 Changes in bovine κ-casein variants (alleles)

Variant/ allele	Position 10	36	97	104	130	135	136	148	148–151	153	155	GenBank accession	SwissProt accession	References
A	**Arg**	**Pro**	**Arg**	**Ser**	**Pro**	**Thr**	**Thr**	**Asp**	**–**	**Ile**	**Ser**	**AY380228**	**P02668**	Robitaille *et al.* (2005)
B							Ile	Ala				AY380229		Robitaille *et al.* (2005)
B2							Ile	Ala		Thr		M36641		Gorodetskii and Kaledin (1987)
C			His				Ile	Ala						Miranda *et al.* (1993)
D			His									AJ619772	Q705V4	Caroli *et al.* (2009)
E											Gly	AF041482		Miranda *et al.* (1993)
F1								Val						Sulimova *et al.* (1992)
F2	His											AF123250		Prinzenberg *et al.* (1996)
G1			Cys			Ile						AF123251		Prinzenberg *et al.* (1996)
G2								Ala				AJ849456 AJ841941	Q5ZET1	Sulimova *et al.* (1996)
H							Ile					AF105260		Prinzenberg *et al.* (1999)
I				Ala								AF121023		Prinzenberg *et al.* (1999)
J							Ile	Ala			Arg			Mahé *et al.* (1999)
K		Leu			Arg			Ala				AH009225		
L								Ala	INS			AY095311		Prinzenberg *et al.* (2008)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

INS: 4 amino acid long insertion, corresponding to a duplication of sequence 148–151 (Ala-Ser-Pro-Glu) of the bovine allele B although 147–150 (Glu-Ala-Ser-Pro) is also possible

(position 10 and 97) or in the appearance (position 130) of a site of cleavage by pancreatic trypsin in the digestive tract. Furthermore, mutations involving T135 (G1) and T136 (B, B2 and C) determine the loss of a potential site of glycosylation. Lastly, the S104A modification occurring in the I variant alters the -Ser-Phe-Met-Ala- chymosin-sensitive site described by Visser *et al.* (1976) and can thus affect the rennet-induced clotting of milk.

15.4.1.5 Bovine β-Lactoglobulin

BLG is the major whey protein in cows' milk. Eleven genetic variants have been reported, with variants A and B being the most frequent (Farrell *et al.*, 2004). The sequence of bovine BLG cDNA (variant A) was established by Alexander *et al.* (1989) and translated into a 162-amino-acid polypeptide containing five cysteines, of which four are involved in intramolecular disulphide bonds. A list of the 13 currently known variants is given in Table 15.5. Variants A and B are common in most cattle breeds (Farrell *et al.*, 2004). BLG variants A and B differ by two amino acid substitutions: Asp64Gly (D64G) and Val118Ala (V118A). Since the last edition of this book, only one novel variant having amino acid substitutions compared to B has been reported and was already included in the lists by Farrell *et al.* (2004) and Caroli *et al.* (2009). It was named W and the authors proposed

Table 15.5 Changes in bovine β-lactoglobulin (BLG) variants (alleles)

Variant/ allele	Position													GenBank accession	SwissProt accession	References
	28	45	50	56	59	64	70	78	108	118	126	129	158			
A						Asp				Val				X14712		Braunitzer *et al.* (1973)
B	**Asp**	**Glu**	**Pro**	**Ile**	**Gln**	**Gly**	**Lys**	**Ile**	**Glu**	**Ala**	**Pro**	**Asp**	**Glu**	Z48305	**P02754**	Braunitzer *et al.* (1973)
B*														DQ489319		Braunschweig and Leeb (2006)
C					His											As reported by Ng-Kwai-Hang and Grosclaude (2003)
D		Gln														As reported by Brignon and Ribadeau-Dumas (1973)
Dr	Asn															As reported by Bell *et al.* (1981a)
E													Gly			As reported by Ng-Kwai-Hang and Grosclaude (2003)
F			Ser									Tyr	Gly			Bell *et al.* (1981a)
G								Met					Gly			Bell *et al.* (1981a)
H						Asp	Asn			Val						Conti *et al.* (1988) and Davoli *et al.* (1987)
I									Gly							Godovac-Zimmermann *et al.* (1996)
J											Leu					Godovac-Zimmermann *et al.* (1996)
W				Leu												Godovac-Zimmermann *et al.* (1990)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

that it could result from an isoleucine to leucine substitution at position 56 (I56L), but this was not further confirmed. This nomenclature is provisionally retained here. Compared to previous reports, variants Dr and B* are included as true protein variants. The first (Dr) is quite unique in that it can be found in a glycosylated form, as described by Bell *et al.* (1970). The authors first described a sequence identity with variant A, but in a subsequent report (Bell *et al.*, 1981a), they mentioned an aspartic acid to asparagine substitution at position 28 (D28N), which would be consistent with the migration pattern observed and with the appearance of a glycosylation site. On the other hand, the product of *BLG* allele *B** (here named variant B*, accordingly) is added to the list; notwithstanding, it has the same primary structure as variant B. In fact, the quantitative polymorphism associated with *BLG*B** (Kim *et al.*, 1996), resulting in 40% of the amount of both transcripts and protein, as compared with the B allele (*BLG*B*), has been resolved recently (Braunschweig and Leeb, 2006). It was well established that the predominant two genetic variants, A and B, are differentially expressed (Cerbulis and Farrell, 1975). Numerous studies on various breeds reported a higher expression of the BLG A variant compared with the B variant. Extensive investigation of the genetic variation in the promoter region of the *BLG* gene revealed the existence of specific haplotypes associated with the A and B variants, respectively. Two SNPs (g.1772G>A and g.3054C>T) lead to amino acid changes (G64D and A118V, respectively) and are the causal genetic polymorphisms of BLG variants B (G64, A118) and A (D64, V118). However, the genetic basis for the differential expression of *BLG A* and *B* alleles still remained elusive. Would the extremely weak BLG B* variant ultimately solve the puzzle? Comparative DNA sequencing of 7,670 bp of the *BLG*B** allele and the established *BLG*B* allele revealed a unique difference of a C to A transversion at position 215 bp upstream of the translation initiation site (g.-215C>A). This mutation segregated perfectly with the differential phenotypic expression. Additional genetic variation further upstream in the 5′-flanking region of the *BLG* gene was identified, including six single-nucleotide substitutions, a single-nucleotide deletion, and a 7 bp duplication. Comparison of DNA sequences showed that the investigated 5′-flanking region is highly conserved between ruminants, and the duplication g.-1885_-1879dupCTCTCGC as well as the substitution g.-1888A>G is found only in the *BLG*A* and *D* alleles in cattle. However, no transcription binding site is predicted to correspond to the region around the duplication (g.−1885_−1879dupCTCTCGC). A cytosine at position g.-1957 and two thymines at positions g.-2008 and g.-2049 were found only in *BLG*B* alleles. It is suggested that the described genetic variability contributes to the differential allelic expression of the *BLG* gene (Braunschweig, 2007). Recently, Ganai *et al.* (2009) detected 50 polymorphisms within the coding, intron and promoter regions of bovine *BLG* gene, of which 42 were in complete linkage disequilibrium (LD) with BLG protein variants A and B. One of the eight polymorphisms remaining (six segregating with variant A and two with variant B) had a significant effect on BLG protein concentration. This SNP, g.-731G>A, segregated only within cows homozygous for BLG variant A. These new reported polymorphisms, including the 7 bp duplication in the *BLG* A 5′-flanking region, may contribute to the allele-specific differential expression of *BLG* (Fig. 15.1). The story is far from being written and the 5′-flanking region and 5′ UTR of the *BLG* gene have to be scanned in additional populations. NGS technologies will probably provide some answers in the near future.

15.4.1.6 Bovine α-Lactalbumin

The primary structure of bovine α-lactalbumin was determined by Brew *et al.* (1970) at the amino acid level and confirmed at the nucleic acid level by Hurley and Schuler (1987) and Wang *et al.* (1989). The mature protein is a polypeptide of 123 amino acid residues, containing eight cysteines that form four intramolecular disulphide bonds. Together with variant B, a variant A (Bhattacharya *et al.*, 1963; Grosclaude *et al.*, 1974) and a variant C (Bell *et al.*, 1981b) were described at the protein level (Table 15.6), the first being present but rare in European cattle and

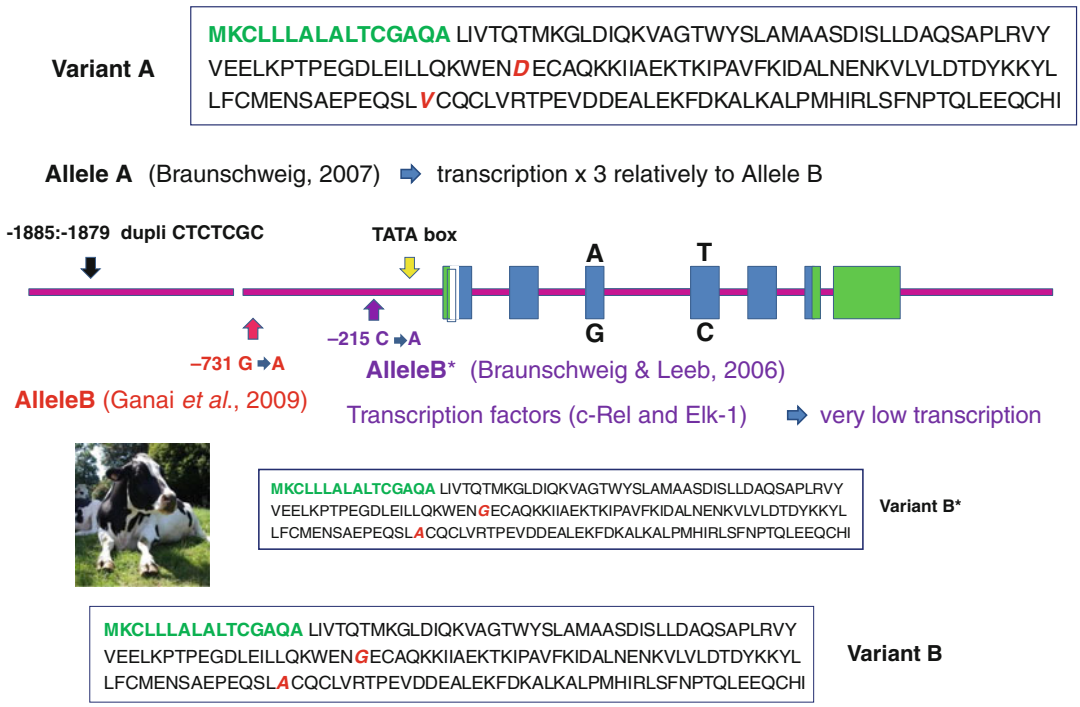

Fig. 15.1 Schematic representation of the bovine β-lactoglobulin gene (*BLG*) and the respective haplotypes for alleles *A*, *B* and *B** in the 5′-flanking region of the transcription unit. Exons are depicted as large *green* (non-coding) and *blue* (coding) boxes. The *white box* codes for the signal peptide. The *yellow arrow* upstream of exon 1 indicates the position of the TATA Box. The upper part of the figure is dedicated to variant A, of which the amino acid sequence is framed and the bottom part to alleles *B* and *B**. The amino acid sequence of the B variant is also given (in smaller frames according to their expression level). The *black arrow* indicates the duplication g.-1885:-1879dupCTCTCGC. SNP g.-731G>A, segregating only within cows homozygous for *BLG*A* (Ganai *et al*., 2009), is indicated by a *pink arrow* whereas the mutation responsible for the very low transcription of allele *B** (g.-215) is indicated by a *violet arrow*

Table 15.6 Changes in bovine α-lactalbumin variants (alleles)

	Position					
Variant/allele	10	?	65	GenBank accession	SwissProt accession	References
A	Gln					Bhattacharya *et al*. (1963)
B	**Arg**	**Asp/Glu**	**Gln**	**M18780**	**P00711**	Brew *et al*. (1970)
				J05147		
C		Asn/Gln[a]	His			Bell *et al*. (1981b)
D	Gln			**JN258330**		Visker *et al*. (2012)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

[a]The authors suggested an Asp to Asn or Glu to Gln substitution in the sequence of the mature protein but could not establish more precisely its position

the last having been observed but not confirmed in Bali cattle. Very recently, a single nucleotide polymorphism g.600G>T was detected in exon 2 of the gene (*LALBA*) coding for α-lactalbumin (Visker *et al*., 2012). This SNP, responsible for the amino acid substitution Gln65His in this new variant (named D), is not expected to affect the protein function.

15.4.2 Caprine Milk Proteins

15.4.2.1 Caprine α_{s1}-Casein

Since the pioneering works by Boulanger *et al*. (1984), goat α_{s1}-casein is known to possess the highest degree of polymorphisms and represents an exceptional paradigm among milk proteins (Grosclaude and Martin, 1997; Bevilacqua *et al*., 2002). The protein was first sequenced by Brignon *et al*. (1989) who found it in a 199 amino-acid (AA) peptide chain, carrying up to 13 potential phosphorylation sites on serine (as well as threonine) residues, of which five are clustered at position 64–68 in the mature protein and does not have any cysteine. Along with its multiple phosphorylation degrees and up to eight so-called *non-allelic variants*, the polymorphism of goat α_{s1}-casein is further increased by the presence of at least 18 alleles encoding several different polypeptide chains (*qualitative polymorphism*). A list of the known variants described up to now is given in Table 15.7. In the case of N, O1, O2, O4-O_N, described as null alleles, the item is entered as allele, in italics, in Table 15.7. The Z variant was predicted only from an isolated transcript and not confirmed at the protein level. It has to be considered very likely as a transcript arising from a cryptic splicing variant, lacking glutamine residue at position 78 and showing two point mutations Ile/Thr and Lys/Asn at position 111 and 114 of the mature protein, respectively. It is provisionally included in italics.

As shown, variants may differ in primary structure (amino acid substitutions), degree of phosphorylation (loss of potential phosphorylation sites) and even length (internally deleted forms). It is still to be ascertained whether the ancestral form is variant B2 (as assumed here) or B1. Most of the other variants may have originated from single-nucleotide mutations leading to amino acid modifications, with the exception of variant M, for which an interallelic recombination between an A and a B2 ancestor has been suggested by Bevilacqua *et al*. (2002). More recently, a similar mechanism has been suggested for the genesis of allele N by Ramunno *et al.* (2005) who described it as the possible result of a recombination event between alleles A and F. Referring to phosphorylation, it can be seen that the substitution Glu to a Gln at position 77, in the A variant (and the derived ones, namely, G, H, I and M), causes the loss of a potential phosphorylation site on Ser75. The mutation Ser/Leu at position 66 causes the loss of another potential site (Ser 64), in variant M. With respect to the chain length, variants G and F were described as deleted variants since they lack, respectively, 13 and 37 amino acid residues, due to exon 4 (G) and exons 9–11 (F) skipping. The D variant has been removed and appears cited in brackets in the table. The genetic study by Mahé and Grosclaude (1989) should be more correctly attributed to variant G. For the F allele, in particular, the deletion of a cytidine in the coding sequence at position 23 of exon 9 is responsible for a complex splicing process which in turn results in the production of an array of RNA forms, five of which appeared to be original F allele, as first reported by Leroux *et al*. (1992). These authors suggested that the previously described variant D (Brignon *et al*., 1990) could be in fact the result of an alternative splicing event of allele F pre-messengers but failed to recover the exactly corresponding RNA. They actually found a transcript form in which exon 9 was lacking as well as Gln78, known to be stochastically lost during the splicing process of primary transcripts. This hypothesis was confirmed (Ramunno *et al*., 2005). Finally, goat *CSN1S1* alleles are associated with at least four levels of α_{s1}-casein synthesis (quantitative polymorphism). Alleles are therefore referred to as "strong" (A, B1, B2, B3, B4, C, H, L, and M), averaging 3.6 g/L per allele; "intermediate" (E and I), yielding 1.6 g/L; "weak" (F and G), with 0.6 g/L; and "null" (N, O1, O2, O_N), leading to non-detectable amounts of α_{s1}-casein in milk. It must be noted that the E and I variants share the same primary sequence as B4 and A, respectively. They are considered as different variants. Nevertheless, it would be more consistent to consider that the relevant alleles (E and B4, as well as A and I) are actually different, showing mutations in non-coding sequences, that translate into the same protein at different expression levels (*quantitative polymorphism*). In the case of the E allele, this phenomenon was

Table 15.7 Changes in caprine α_{s1}-casein variants (alleles)

Variant/ allele	Position 1	8	14–26	16	59–69	59–95	64	66	75	77	78	90	100	111	114	195	GenBank accession	SwissProt accession	References
A				Leu					Ser	Gln							AJ504710	Q8MIH4	Brignon *et al.* (1989) and Ramunno *et al.* (2004)
B1				Leu															Grosclaude *et al.* (1994) and Grosclaude and Martin (1997)
B2	**Arg**	**His**		**Pro**			**SerP**	**SerP**	**SerP**	**Glu**	**Gln**	**Arg**	**Arg**	**Ile**	**Lys**	**Thr**		**P18626**[a]	Grosclaude *et al.* (1994) and Grosclaude and Martin (1997)
B3													Lys						Grosclaude *et al.* (1994) and Grosclaude and Martin (1997)
B4													Lys			Ala			Grosclaude *et al.* (1994) and Grosclaude and Martin (1997)
C		Ile											Lys			Ala			Brignon *et al.* (1989)
(D)[b]					DEL														Ramunno *et al.* (2005) and Brignon *et al.* (1990)
E													Lys			Ala	X72221[c]		Jansà-Perez *et al.* (1994)
F						DEL											AJ504711	Q8MIH3	Leroux *et al.* (1992) and Ramunno *et al.* (2005)
G			DEL						Ser	Gln									Mahé and Grosclaude (1989) and Martin and Leroux (1994)
H	Lys			Leu					Ser	Gln									Chianese *et al.* (1997a, b)
I				Leu					Ser	Gln									Chianese *et al.* (1997a, b)
L												His							Chianese *et al.* (1997a, b)
M							Ser	Leu	Ser	Gln									Bevilacqua *et al.* (2002)
N						Δ											*AJ504712*	*Q8MIH2*	Ramunno *et al.* (2005)
O1																	*AJ252126*		Cosenza *et al.* (2003)
O2																			Martin *et al.* (unpublished)

(continued)

Table 15.7 (continued)

Variant/ allele	Position 1	8	14–26	16	59–69	59–95	64	66	75	77	78	90	100	111	114	195	GenBank accession	SwissProt accession	References
O $_N$													Δ						Ådnøy *et al*. (2003) and Hayes *et al*. (2006)
Z											DEL			Thr	Asn		AY344966	Q69EZ6	

Variants are presented in different rows; *null alleles* are presented in italics; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

DEL: internal deletion of the corresponding sequence in the mature protein

Δ refers to a nucleotide deletion occurring in exons 9 and 12

[a]UniProt accession number P18626 refers to variant B2 except for a sequence conflict at position 9 (R/Q) in the mature protein. To our knowledge, this conflict is inconsistent with the available literature. All the published sequences show a Q residue at position 9

[b]Predicted form of alternatively spliced transcripts arising from the F allele, it is not a genetic variant

[c]There is a sequence conflict (T/A) regarding the amino acid residue at position 194 in the mature protein due to a mistake in the nucleotide sequence given in GenBank (accession number X7222) referring to allele E, codon 209 should be ACT (Thr) instead of GCT (Ala)

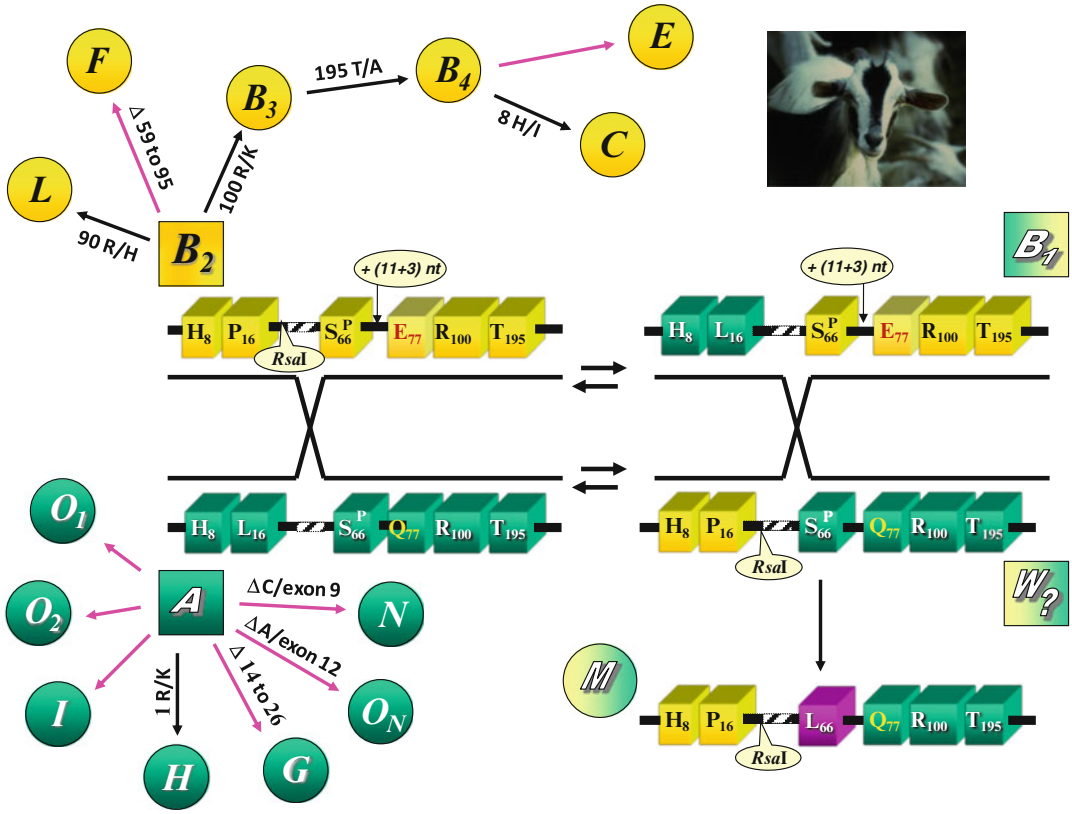

Fig. 15.2 A putative phylogeny integrating a possible interallelic recombination between two allelic lineages of α_{s1}-casein (adapted from Bevilacqua *et al*., 2002). Four alleles (B2, A, B1, and W) putatively involved in a recombination event are schematically represented as a chain of six boxes (exons) on which are indicated polymorphic amino acid residues and their position in the peptide chain, thus providing a simplified haplotype formula (e.g. HPS^{P}ERT and HLS^{P}QRT for alleles B2 and A, respectively). The *Rsa*I polymorphic restriction site and insertions occurring between exons 6 and 8 and within intron 9, respectively, are indicated. Alleles deriving from these four potentially recombinant alleles (*boxed*) are *circled*. *Arrows* indicate a possible pathway of evolution to alleles associated with high (*black*) or reduced (*pink*) amounts of casein synthesised. The M allele is derived from allele W by a single-nucleotide transition C>T (nucleotide 23/exon 9) leading to the occurrence of a Leu residue (allele M) instead of the Ser (putative allele W) in the multiple phosphorylation site of the protein. This new phylogeny has been enriched with three novel variants (H, I and L) reported by Chianese *et al.* (1997a, b) and alleles N (Ramunno *et al.*, 2005) and O_N, the Norwegian "Null" allele (Ådnøy *et al*., 2003)

explained as the result of the insertion of a long interspersed repeated element (LINE) sequence in the 3′ UTR that is thought to reduce the stability of mRNA (Jansà-Perez *et al*., 1994). A similar mechanism is supposed to explain the reduced expression rate of allele I, but has not been proved to date. Interestingly, the F allele is a model of both qualitative (internal deletion) and quantitative (rate of expression) polymorphism, as well as of non-allelic variants. Furthermore, it could be speculated that allele N, for which a large population of transcripts (as the results of alternative splicing events) was actually detected (Ramunno *et al.*, 2005), amounting to 1/3 those of the F variant (as total RNA concentration), could be in fact a "false null" allele and one or more truncated forms could be present in milk at hardly detectable levels. It seems that allele *CSN1S1*N* could be the counterpart of *CSN1S1*F* from the allelic lineage A (Fig. 15.2). As far as *CSN1S1*O*$_N$ is concerned, the mutation responsible for the very low expression, if any, of which the frequency is

very high (ca. 80%) in the Norwegian dairy goat population (Ådnøy *et al*., 2003), is a single-nucleotide deletion occurring in exon 12. This deletion results in the occurrence of a premature stop codon leading to a theoretical truncated protein made of 137 amino acid residues which remains to be found. Given its allele haplotype, it may originate from the A lineage.

15.4.2.2 Caprine β-Casein

The goat β-casein gene (*CSN2*), first sequenced by Roberts *et al*. (1992), translates into a 222-AA precursor which, after cleavage of the signal peptide, is secreted as a 207 AA polypeptide chain. It may carry up to five phosphoserine residues (four of which are clustered at positions 15, 17, 18 and 19) and two phosphothreonine residues and has no cysteine. Table 15.8 summarises the known protein variants and alleles. Compared to α_{s1}-casein, the protein is thought to possess a lower degree of polymorphism, since only eight alleles have been characterised so far, two of which were originally described as "null" alleles. The B variant was the first to be described (Mahé and Grosclaude, 1993), together with a null allele (*CSN2*O*). The authors did not determine its primary structure, but speculated that the B variant could differ from A by an additional phosphate group. Keeping in mind that the protein is present mainly in its 6 and 5 phosphate forms, it still remains uncertain whether the B variant originates from a mutation leading to one more potential phosphorylation site or is merely an A variant at its maximum degree of phosphorylation. The genomic DNA sequence of a novel allele was first submitted by Wang *et al*. (accession number AF409096). The primary structure and phosphorylation sites of the corresponding protein were subsequently given by Neveu *et al*. (2002a), who named it variant C. A non-allelic variant D was described by Galliano *et al*. (2004) in homozygous C goats at the *CSN2* locus and probably originated from an incorrect translation of the genetic information, leading to an Asn instead of a Val residue at position 207. Caroli *et al*. (2006), in a study on casein genetic polymorphisms in local Italian breeds, identified a novel mutation at the *CSN2* locus by PCR-SSCP, which translated into a variant, named E, differing from variant A by the substitution Ser/Tyr at position 166 of the mature protein. Lately, Moatsou *et al*. (2007) described a novel variant showing a Tyr instead of an Asp residue at position 47. The authors also named this variant E. Here we propose to name it *CSN2*F* in order to avoid any possible ambiguity and to respect the chronological order of identification. As mentioned above, the first evidence of milk samples lacking the electrophoretic band of β-casein was provided by Mahé and Grosclaude (1993) who also suggested the existence of two alleles controlling this phenomenon in the Creole population analysed. Two distinct alleles were then described by Persuy *et al*. (1996, 1999) and by Rando *et al*. (1996) that could be associated with the absence of this protein in milk. They were named, respectively, *CSN2*O1* and *CSN2*O2*. In the *CSN2*O1* allele, the deletion of a single nucleotide (A residue) in a AAAA sequence between residues 16 and 19 of exon 7 causes a frameshift resulting in a premature stop codon in the coding sequence of the cDNA (codon 73). In the *CSN2*O2* allele, two single point mutations were identified: the first one is a T to C transition at nucleotide −388 from the start site of transcription and the second is a C to T transition at position 373 of exon 7, creating a premature stop codon in the coding sequence (codon 182) of the cDNA (Fig. 15.3). A 20-fold reduction in the quantity of mRNA was observed with *CSN2*O1* (Persuy *et al*., 1999), whereas a tenfold reduction was observed with *CSN2*O2* (Ramunno *et al*., 1995). Despite the marked reduction in the amount of mRNA, Ramunno *et al*. (1995) found that milk from homozygous O2/O2 goats contained ca. 1.6% of β-casein, compared to 53% in the milk from homozygous goats A/A at the *CSN2* locus. Consistent with this result, Cunsolo *et al*. (2006) combining HPLC and MS/MS techniques, identified and sequenced a 166 AA protein whose sequence corresponds to the truncated protein theoretically encoded by the *CSN2*O2* allele. Recently, a new *CSN2* allele (here named *CSN2*O3*) showing a SNP (g1311T>C) in the promoter region has been reported as being associated with the absence of β-casein in milk (Cosenza *et al.*, 2007). In fact,

Table 15.8 Changes in caprine β-casein variants (alleles)

Variant/allele	Position							
	47	166	167–207	177	207	GenBank accession	SwissProt accession	References
A	**Asp**	**Ser**		**Ala**	**Val**	**AH001195.1**	**P33048**	Roberts *et al.* (1992)
B								Mahé and Grosclaude (1993)
C				Val		AF409096		
(D)[a]				Val	Asn			Galliano *et al.* (2004)
E		Tyr						Caroli *et al.* (2006)
F	Tyr							Moatsou *et al.* (2007) and Chianese *et al.* (2007a)
O1						*AF172260*		Mahé and Grosclaude (1993) and Persuy *et al.* (1999)
O2			DEL			AJ011019		Rando *et al.* (1996) and Cunsolo *et al.* (2005)
O3						*AJ011018*		Cosenza *et al.* (2007)

Variants are presented in different rows; *null alleles* are presented in italics; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

DEL: internal deletion of the corresponding sequence in the mature protein

[a]Observed as a non allelic variant from homozygous goat C/C at the *CSN2* locus

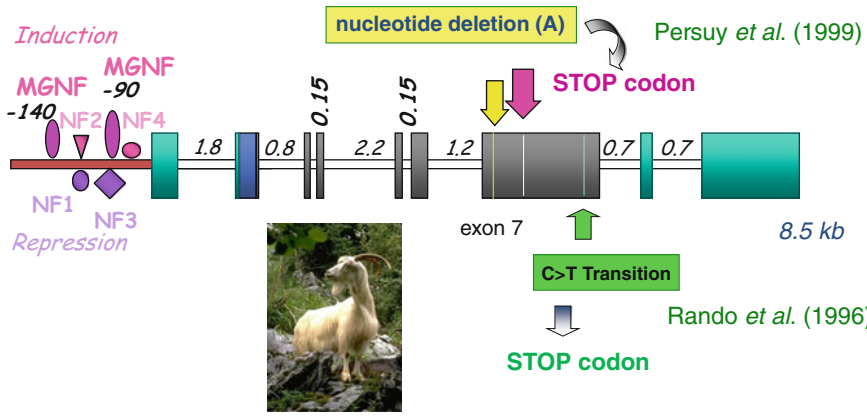

Fig. 15.3 Schematic representation of the goat β-casein encoding gene (*CSN2*). Two distinct mutations responsible for the occurrence of premature STOP codons and consequently for the absence of β-casein in goat milk have been described. The first one (Persuy *et al*., 1999) is due to a single-nucleotide deletion in the 5′ part of exon 7 (*yellow arrow*) whereas the second one (Rando *et al*., 1996) occurring in the 3′ end of the same exon is due to a C>T transition (*green arrow*). Nuclear regulatory factors and their interaction sites with DNA in the 5′ flanking region of the transcription unit are schematised

this SNP corresponds to the first mutation, T to C transition at nucleotide −388, previously reported by the same group. Therefore, one can expect that this single SNP in the promoter region, alone in itself, accounts for the absence of β-casein in milk.

15.4.2.3 Caprine α_{s2}-Casein

The goat α_{s2}-casein was first sequenced by Bouniol (1993) at the cDNA level. The mature protein has 208 AA residues and the first detailed data on the genetic polymorphism were reported by the same group (Bouniol *et al*., 1994). Of the two alleles, A and B, previously described at the goat *CSN1S2* locus, the former was resolved into two alleles, named *CSN1S2*A* and *CSN1S2*C*. α_{s2}-Casein C, which cannot be distinguished from α_{s2}-casein A by starch or polyacrylamide gel electrophoresis, was shown to differ by a single substitution Lys/Ile at position 167 of the mature protein. Goat α_{s2}-casein, like its bovine counterpart, is the most phosphorylated casein with up to 17 potential phosphorylation sites (11 serine and 6 threonine residues), nine of which occur in three clusters regularly distributed on the peptide chain at position 8SerP-SerP-SerP10, 57SerP-SerP-SerP59 and 130SerP-ThrP-SerP132 of the mature protein. Moreover, it contains two cysteine residues that are available for intermolecular disulphide bridges. At present, a total of seven *CSN1S2* alleles have been described in the goat species (Table 15.9). Most of them differ by single-nucleotide substitutions in the coding sequence with the exception of alleles *CSN1S2*D* and *E*. *CSN1S2*D* apparently associated with decreased synthesis of the protein is characterised by an internal deletion spanning over 106 nucleotides, involving the last 11 nucleotides of exon 11 and the first 95 nucleotides of the following intron. This large deletion triggers a deep rearrangement of the messenger in which codon 121 is modified to code for an Asn residue (instead of a Thr residue in variant A), and the following three codons (122–124) are lost (Ramunno *et al*., 2001a, b). In the case of variant E, conflicting sequences have been reported for the mRNA (GenBank accession number AJ249995) and the joined coding DNA (GenBank accession numbers AJ242526, AJ242527, AJ242528, AJ242533, AJ242728, AJ242866, AJ249995, AJ297310, AJ297311, AJ297312, AJ297313, AJ297314, AJ297315, AJ297316, AJ298297) published by Lagonigro *et al*. (2001), and the possible amino acid modifications are

Table 15.9 Changes in caprine α_{s2}-casein variants (alleles)

	Position										GenBank	SwissProt	
Variant/allele	7	62	64	110–208	121	122–124	144	146	167	193	accession	accession	References
A	**Val**	**SerP**	**Glu**		**Thr**		**SerP**	**Glu**	**Lys**	**Pro**	**X65160.1**	**P33049**	Bouniol (1993)
B		Ser	Lys										Bouniol *et al.* (1993b)
C									Ile		S74171		Bouniol *et al.* (1994)
D					Asn	DEL					AJ238684	Q9XSL1	Ramunno *et al.* (2001a)
(E)[a]							(Ser)	(Lys)	Ile	Arg	AJ249995.1		Lagonigro *et al.* (2001)
F	Ile										AJ289716.1		Ramunno *et al.* (2001b)
O				*DEL*							*AJ289715.1*		Ramunno *et al.* (2001a)

Variants are presented in different rows; the *null allele* is presented in italics; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

DEL: internal deletion of the corresponding sequence in the mature protein

[a]Conflicting sequences concerning the Glu to Lys modification at position 146 were reported for mRNA and genomic DNA

provisionally presented into brackets. In the case of the null allele (*CSN1S2*O*), a transversion G to A occurs at the 80th nucleotide of exon 11, giving rise to a premature stop codon in the cDNA sequence (codon 110). A truncated protein of 109 AA could therefore be predicted, but none has been detected in milk up to date.

15.4.2.4 Caprine κ-Casein

Before the last edition of *Advanced Dairy Chemistry-1: Proteins* in 2003, κ-casein was thought to be scarcely polymorphic in the goat species, as very little evidence had been found for amino acid modifications or differences in electrophoresis patterns. Nevertheless, over the last 10 years, the systematic use of molecular biology approaches revealed an array of genetic polymorphisms, many of which translated into different protein variants. Most of these new developments have been reviewed by Prinzenberg *et al*. (2005) who proposed a new nomenclature basically according to the chronological order of GenBank accession numbering. Table 15.10 shows the variants detected to date in the goat species. Applying the principle proposed by Prinzenberg *et al*. (2005), the nomenclature was revised and variant L which is actually a different allele but coding for the same protein variant as G was suppressed, and the successive variants have been renamed accordingly.

Caprine κ-casein cDNA was first sequenced by Coll *et al.* (1993). The mature protein is a 171-amino-acid chain (two residues more than its bovine counterpart) with three serine and one threonine residues being potentially phosphorylated. It has two cysteine residues at positions 10 and 11 that are available for the formation of intermolecular disulphide bridges with other κ-casein or α_{s2}-casein molecules. Moreover, in analogy with the other κ-casein, it can have various degrees of glycosylation.

Regarding its genetic polymorphism, since the publication of the above-mentioned review by Prinzenberg *et al*. (2005), a systematic sequencing of exon 4 made it possible to identify seven new alleles in Indian Jakhrana goats (Gupta *et al*., 2009) and four in goat populations from East Africa (Kiplagat *et al*., 2010). The predicted primary structures of such alleles, however, do not always result in new protein variants, and therefore, the nomenclature proposed by these authors was modified accordingly to follow the chronological order of publication. Regarding the new variants reported by Gupta *et al.* (2009), several differences could be found between sequences provisionally submitted to GenBank and those presented and discussed later in the cited paper. In case of conflict, the amino acid substitutions given in Gupta *et al.* (2009) were preferred.

15.4.2.5 Caprine β-Lactoglobulin and α-Lactalbumin

To date, no protein polymorphisms have been described for these whey proteins in the goat species, with the exceptions of isolated observations by Boulanger (1976) and Russo *et al*. (1986), respectively, on BLG and α-lactalbumin. These findings, however, did not lead to the characterisation of new variants nor were they confirmed in following studies.

15.4.3 Ovine Milk Proteins

15.4.3.1 Ovine α_{s1}-Casein

Ovine α_{s1}-casein was first sequenced by Ferranti *et al*. (1995). It is a 199 AA-residue polypeptide with up to ten potentially phosphorylated serine residues displaying a 97% identity with the primary structure of its caprine counterpart. The two proteins share a common feature: a high molecular diversity in part due to alternative splicing variants. Nevertheless, unlike the goat, the existence of quantitative polymorphisms or null alleles has not been proved so far in the ovine species, although Giambra *et al.* (2010a) reported recently an average 26% reduction in the expression level of the *CSN1S1*H* allele. The first variants to be described, A, B, C, D and E, migrate with decreasing speed on basic electrophoresis gels and show the opposite behaviour in acidic capillary electrophoregrams (as reported by Amigo *et al*., 2000). With the exception of variant B, of which the primary structure remains to be determined (but for which the modification of an acidic AA to a neutral one can be hypothe-

Table 15.10 Changes in caprine κ-casein variants (alleles)

Variant/ allele	Position										GenBank accession	SwissProt accession	References
	44	53	61	65	77	90	119	145	156	159			
A	**Gln**	**Asn**	**Tyr**	**Val**	**Gln**	**Asp**	**Val**	**Val**	**Ala**	**Ser**	**X60763** **EF053353**	**P02670**	Coll *et al.* (1993), Gupta *et al.* (2009) and Kiplagat *et al.* (2010)
B							Ile				AF485340 AF434988 AY166705 AY166706 AY166707	Q540J1	Yahyaoui *et al.* (2001), Jann *et al.* (2004) and Kiplagat *et al.* (2010)
C				Ile			Ile		Val	Pro	AY350425 AF485341		Yahyaoui *et al.* (2001) and Prinzenberg *et al.* (2005)
D	Arg			Ile			Ile			Pro	AY027868 AY090465		Caroli *et al.* (2001) and Yahyaoui *et al.* (2001)
E						Gly	Ile				AF486523		Angiolillo *et al.* (2002)
F							Ile			Pro	AY090466		Yahyaoui *et al.* (2003)
G				Ile			Ile			Pro	AY090467 AY166708		Yahyaoui *et al.* (2003) and Jann *et al.* (2004)
H		Ser					Ile				AF521022		Jann *et al.* (2004)
I				Ile			Ile				AY166710		Jann *et al.* (2004)
J			Cys				Ile				AY166711		Jann *et al.* (2004)
K	Arg						Ile				AY166709 EF053350		Jann *et al.* (2004)
L						Asn	Ile	Ala		Pro	AY428577		Prinzenberg *et al.* (2005) and Kiplagat *et al.* (2010)
M	Arg	Ser			Arg		Ile				EF053351 EF053354		Gupta *et al.* (2009)
N	Arg										EF053352		Gupta *et al.* (2009)
O		Ser								Pro	EF053355		Gupta *et al.* (2009)
P		Ser			Arg						EF053356		Gupta *et al.* (2009)
Q				Ile						Pro			Kiplagat *et al.* (2010)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column
DEL: internal deletion of the corresponding sequence in the mature protein

Table 15.11 Changes in ovine α_{s1}-casein variants (alleles)

Variant/allele	Position 12	13	43–50	51–58	64	66	68	70–77	194	GenBank accession	SwissProt accession	References
A	SerP	Ser									P04653	Ferranti *et al.* (1995)
B												Chianese *et al.* (1996)
C′									Thr	AY444506		Ceriotti *et al.* (2004, 2005)
C	**Ser**	**Pro**			**SerP**	**SerP**	**SerP**		**Ile**	**X03237**[a]		Ferranti *et al.* (1995)
D					Ser	Ser	Asn					Ferranti *et al.* (1995)
E					Ser	Ser	Ser	DEL				Chianese *et al.* (1996, 2007b)
F												Pirisi *et al.* (1999)
G												Chianese (unpublished)
H				DEL						FJ440846		Giambra *et al.* (2010a)
I			DEL							FJ695513 FJ695515		Giambra *et al.* (2010b)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column
DEL: internal deletion of the corresponding sequence in the mature protein
[a]The original mRNA sequence refers to the alternative spliced variant lacking exon 16, coding for a 191 AA residues internally deleted protein, lacking residues 141 to 148

sised), protein sequencing of the other four variants made it possible to explain this variability with a progressive loss in potential phosphorylation sites, as shown in Table 15.11. In particular, the substitution Ser/Pro at position 13 would interfere with the phosphorylation of Ser12 in variant C. Variant D and E lack, respectively, 2 and 3 phosphoserine residues in the phosphorylation cluster at position 64–68 due to an amino acid substitution at position 68 and the deletion of eight AA residues (encoded by exon 8) between positions 70 and 77, respectively. Recently, Ceriotti *et al*. (2004, 2005) found and characterised a novel allele (named *CSN1S1*C′*) coding for a protein that differs from the C variant by a single-AA substitution, Ile/Thr, at position 194 (and not at position 186, as mentioned erroneously in the original manuscript by Ceriotti *et al*., (2004), according to the published sequence. The authors suggested that this could be the ancestral form in the ovine species because a Thr residue is conserved at the same position both in goat and in cattle. Two novel variants named F and G were found, respectively, by IEF and RP-HPLC techniques, but their primary structure has not yet been published. More recently, two novel variants, named H and I, were identified by Giambra *et al.* (2010a, b) and characterised at the protein and nucleotide levels. Both are described as internally deleted proteins lacking the amino acid sequence encoded by exon 8 and exon 7, respectively. Variant I, thus, loses two potential phosphorylation sites encoded by exon 7.

15.4.3.2 Ovine β-Casein

The sequence of ovine β-casein, first proposed by Richardson and Mercier (1979) according to automated and manual Edman degradation sequencing of acid and enzymatic hydrolysates, was later corrected by Provot *et al.* (1989), who sequenced the cDNA and the gene (Provot *et al*., 1995). Similar to the caprine protein, the precursor of ovine β-casein is a 222 AA-residue polypeptide, made of a 15 AA-signal peptide and a 207 AA-mature protein. Potential phosphorylation sites are the same as in the goat protein, and ovine β-casein likewise lacks cysteine residues. The known variants are presented in Table 15.12. Chianese (1997) first identified two variants, named B and C, by IEF, but only the sequence of variant C is presently available. It contains a Gln instead of a Glu residue at position 2. Ceriotti *et al*. (2004) and Chessa *et al*. (2010) identified two polymorphic patterns within exon 7 by PCR-SSCP and DNA sequencing that lead up to two novel protein variants, here named D and E, respectively. Compared to the reference variant, the first one has a Val residue instead of a Met at position 183; in the latter, the Leu residue at position 196 is replaced by an Ile residue.

15.4.3.3 Ovine α_{s2}-Casein

In the case of ovine α_{s2}-casein, a series of sequence conflicts and inconsistent nomenclature make the identification of variants difficult. Therefore, to clarify the situation, a new nomenclature is proposed in the present chapter, following the chronological order in which each variant was detected, to unambiguously describe the state of the art regarding the genetic polymorphism of this protein.

A first sequence of ovine α_{s2}-casein was provided by Boisnard and Petrissant (1985) based on mRNA (GenBank accession number, X03238.1; UniProt accession number, P04654). Compared to the bovine and caprine sequences (both having an Asn residue at position 49), the sequence by Boisnard and Petrissant (1985) has an Asp residue at this position, whereas all of the sequences published later have an Asn residue instead. It is therefore referred to as variant A′ in Table 15.13 and will not be considered as the reference sequence hereafter. The actual sequence of variant A differs from that of variant A′ solely for the Asn49 residue, and the genomic DNA sequence corresponding to *CSN1S2*A* allele is now available in GenBank (Giambra *et al*., in press). In a subsequent report by Boisnard *et al*. (1991), an allelic variant, here named B, was identified at the transcript level and characterised by a Lys to Asn substitution at position 200. This mutation was further confirmed by Chessa *et al*. (2010) by sequencing of exon 16. One more protein variant (here named C) was described by Chianese *et al.* (1993) applying an extensive one- and two-dimensional electrophoresis approach, combined

Table 15.12 Changes in ovine β-casein variants (alleles)

	Position					
Variant/allele	2	183	196	GenBank accession	SwissProt accession	References
A	**Glu**	**Met**	**Leu**	**X16482** **X79703**	**P11839**	Provot *et al*. (1989, 1995)
B						Chianese (1997)
C	Gln					Chianese (1997)
D		Val		AY444504		Ceriotti *et al*. (2004)
E			Ile			Chessa *et al*. (2010)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

Table 15.13 Changes in ovine α_{s2}-casein variants (alleles)

	Position								GenBank	SwissProt	
Variant/allele	45	46	48	49	75	105	161	200	accession	accession	References
A′				Asp					X03238.1	P04654	Boisnard and Petrissant (1985)
A	**Val**	**Arg**	**Ala**	**Asn**	**Asp**	**Ile**	**Arg**	**Asn**	**GU169085**		Boisnard *et al.* (1991) and Giambra *et al.* (in press)
B								Tyr			Boisnard *et al.* (1991) and Chessa *et al.* (2010)
C											Chianese *et al.* (1993) and Recio *et al.* (1997b, c)
D					Tyr	Val			GU169086		Chessa *et al.* (2003), Picariello *et al.* (2009a) and Giambra *et al.* (in press)
E	Ile		Ser(P)						GU169087		Giambra *et al.* (in press)
F		Ser							GU169088		Giambra *et al.* (in press)
G							His		GU169089		Giambra *et al.* (in press)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

Table 15.14 Changes in ovine κ-casein variants (alleles)

Variant allele	2	7	104	GenBank accession	SwissProt accession	References
A′	Gln	Glu				Jolles *et al.* (1974a, b)
A	**Glu**	**Gln**	**Ser**	**X51822**	**P02669**	Furet *et al.* (1990)
B			Leu	AY444505		Ceriotti *et al.* (2004)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

Table 15.15 Changes in ovine β-lactoglobulin variants (alleles)

Variant allele	20	148	GenBank accession	SwissProt accession	References
A	Tyr				Ali *et al.* (1990)
B	**His**	**Arg**	**X12817**	**P67976**	Gaye *et al.* (1987)
C		Gln			Erhardt (1989a)

Variants are presented in different rows; amino acids in the *reference variant* are in boldface; amino acid modifications are given in the relevant column

with immunoblotting experiments, and was subsequently confirmed by Recio *et al.* (1997c). The authors named the variant "fast" due to its marked anodic mobility. They concluded that it should be an internally deleted variant with a greater negative net charge and lower isoelectric point, but no further characterisation was performed. Later, Chessa *et al.* (2003) detected a novel protein variant showing higher isoelectric point than variant A on IEF gels and named it B (here renamed D). Picariello *et al.* (2009a), using MALDI-TOF MS and MALDI-PDS-TOF MS, were able to identify two amino acid substitutions, Ile for Val at position 105 and Asp for Tyr at position 75, the latter being responsible for the newly observed IEF pattern. As mentioned above, it is only recently that Giambra *et al.* (in press) were able to confirm the genomic DNA sequences of variants A (Asn49) and D (Asn49, Tyr75, Val105) (according to the new nomenclature proposed) and describe three novel variants (here named E, F and G).

15.4.3.4 Ovine κ-Casein

The first sequence of ovine κ-casein (named A′ in Table 15.14) was published by Jolles *et al.* (1974a, b) for *para*-κ-casein and for the caseinomacropeptide, respectively, based primarily on trypsin digestion of the mature protein and direct N-terminal sequencing of peptides. Later, Furet *et al.* (1990) reported a cDNA sequence which therefore included the signal peptide. Like its caprine counterpart, mature ovine κ-casein is a 171 residue polypeptide with four potential phosphorylation sites (three serine and one threonine), two cysteine residues and several potential *O*-glycosylation sites. Unlike its bovine and caprine counterparts, the ovine protein appears to be scarcely polymorphic, since only a single variant, detected on genomic DNA by PCR-SSCP, has been described so far (Ser to Leu substitution, at position 104) by Ceriotti *et al.* (2004).

15.4.3.5 Ovine β-Lactoglobulin

Ovine BLG is a 162 amino-acid polypeptide. Since the work of Bell and McKenzie (1967), two variants of ovine BLG are known and named A and B. The cDNA sequence for variant B was published by Gaye *et al.* (1987), and the mutation responsible for the His to Tyr substitution at position 20 in variant A was reported by Ali *et al.* (1990), whereas a novel protein variant (named C) described by Erhardt (1989a) was sequenced (Prinzenberg and Erhardt, 1999). The three variants are presented in Table 15.15.

15.4.3.6 Ovine α-Lactalbumin

Two nucleotide sequences are available for the ovine α-lactalbumin on GenBank (accession numbers X06367 and AB052168), the latter

being taken as a reference in the UniProt database (accession number P09462). However, although the two predicted sequences differ for four amino acid residues, they were not attributed to any of the two known variants observed so far, named A and B (Schmidt and Ebner, 1972).

15.5 Genetic Polymorphism in Milk-Fat Globule Membrane Proteins

The secretion process of milk lipids initiates in the endoplasmic reticulum with the budding of lipid droplets. These lipid droplets then migrate to the apical pole of the mammary epithelial cell (MEC), where they are progressively enveloped by the plasma membrane and released as fat globules in the milk (Mather and Keenan, 1998). Hence, most of MFGM-associated proteins belong to the ER and plasma membrane compartments. In addition, cytoplasmic inclusions, known as "crescents", are often trapped between the outer membrane and the lipid core during the secretion process (Wooding *et al*., 1970). These crescents contain soluble, cytoplasmic proteins which can be also identified in MFGM isolates.

Biochemical approaches (SDS-PAGE followed by diverse staining procedures) or molecular biology techniques have been initially employed to characterise MFGM proteins. Roughly, MFGM material can be resolved by SDS-PAGE into eight protein bands corresponding to MUC-1 (mucin-1), fatty acid synthase (FAS), xanthine oxidoreductase (XOR), MUC 15 (PASIII), CD36, butyrophilin (BTN1A1), milk-fat globule EGF factor 8 (MFG-E8) or lactadherin (LDH), and adipophilin (ADRP, adipose differentiation-related protein). Major MFGM proteins have been reviewed extensively (Mather, 2000). Historically, two-dimensional gel electrophoresis coupled to mass spectrometry methods was used to identify MFGM-associated proteins (Fortunato *et al*., 2003; Fong *et al*., 2007; Barello *et al*., 2008). However, these methods are not suited to analyse large hydrophobic proteins as found in MFGM. Recent advances in the field of proteomics, including development of one-dimensional gel electrophoresis approach coupled to tandem mass spectrometry (GeLC-MS-MS), led to the identification of hundreds of proteins associated to the MFGM in several species, including humans (Hettinga *et al*., 2011), cattle (Reinhardt and Lippolis, 2006) and sheep (Pisanu *et al*., 2011). Thus, the number of MFGM proteins, as well as biological functions associated with MFGM proteins (including fat metabolism, cell trafficking or signalling, or immune-related functions), is still growing.

15.5.1 Genetic Polymorphisms Associated with MFGM Protein-Encoding Genes

Genetic polymorphisms within MFGM protein-encoding genes reported to date are summarised in Table 15.16. As expected, since MFGM proteins are involved in lipid secretion processes, most of reported genetic polymorphisms are associated with dairy traits related to quantitative (milk-fat yield) or qualitative (fatty acid composition) aspects of fat in milk.

15.5.1.1 MUC-1

Mucins are large proteins containing more than 50% carbohydrates by weight which are present at the interface between epithelia and their extracellular environment. They play an essential role in forming protective mucous barriers on epithelial surfaces. MUC-1 is undoubtedly the best characterised milk mucin, and, historically, MUC-1 polymorphism is probably the earliest genetic polymorphism reported to date for MFGM proteins.

Bovine MUC-1 is a highly glycosylated protein of 580 amino acid residues comprising a signal peptide of 22 residues and encoded by a rather compact gene (ca. 4 kb), known to contain a highly polymorphic variable number of tandem repeats (VNTR) domain. General features of the protein are a large extracellular region with highly conserved tandem repeats of 20 amino acids

Table 15.16 Genetic polymorphisms identified within milk-fat globule membrane protein-encoding genes and associated dairy traits

MFGM protein-encoding gene	Species	Associated dairy trait	References
MUC-1	*Bos taurus*, *Ovis aries*, *Capra hircus*, *Bos grunniens* (yak)	Milk protein and fat percentages (weak)	Patton and Patton (1990), Hens *et al.* (1995), Jiang *et al.* (2004), Sacchi *et al.* (2004), Rasero *et al.* (2007) and Sando *et al.* (2009)
FASN	*Bos taurus*	Milk fatty acid composition	Roy *et al.* (2006), Morris *et al.* (2007) and Schennink *et al.* (2009)
BTN1A1	*Bos taurus*, *Bubalus bubalis* (water buffalo), *Ovis aries*, *Capra hircus*	Milk yield, milk fat yield, total solids	Bhattacharya *et al.* (2007) and Qu *et al.* (2010)
MFGE-8	*Capra hircus*	Milk fat yield, total solids	Qu *et al.* (2010)
ABCG2	*Bos taurus*, *Bos indicus* (zebu), *Bubalus bubalis*	Milk yield, fat and protein percentages	Cohen-Zinder *et al.* (2005), Ron *et al.* (2006), Tantia *et al.* (2006), Olsen *et al.* (2007) and Yue *et al.* (2011)
FABP	*Ovis aries*	Fat contents	Calvo *et al.* (2004)

MUC-1 mucin-1; *FASN* fatty acid synthase; *BTN* butyrophilin; *MFG-E8* milk fat globule EGF factor 8, lactadherin; *ABCG2* ATP-binding cassette subfamily G, member 2; *FABP* fatty acid-binding protein, heart-type

(VNTR domain), a membrane-proximal SEA (sperm protein, enterokinase, and agrin) module, which is a 120 amino-acid domain frequently associated with heavily *O*-glycosylated proteins, a transmembrane region and a short (70 amino acids) cytoplasmic tail (Pallesen *et al*., 2001).

Because each codominant inherited allele may contain a variable number tandem repeat (VNTR) encoding the 20-amino-acid motif, different sizes of MUC-1 are observed by SDS-PAGE analysis. Heterozygous individuals display two bands for MUC-1 protein in SDS-PAGE whereas a single band is observed for homozygous individuals. Genetic polymorphism of *MUC-1* has been demonstrated for several dairy species, including bovine (Patton and Patton, 1990), caprine (Sacchi *et al*., 2004; Cebo *et al*., 2010) and ovine (Rasero *et al*., 2007) species. It has been demonstrated that the number of tandem repeats vary between species as well between breeds. Indeed, up to five alleles were reported in a Holstein population of dairy cows whereas a reduced number of *MUC-1* alleles was found in Ayrshire, Jersey, Italian Friesian and Piedmontese or Brown Swiss cattle (Huott *et al*., 1995; Sacchi *et al*., 1995; Rasero *et al*., 2002). The existence of 15 different alleles, showing a repetitive region ranging in size between 1,500 and 3,000 bp, has been reported in the goat MUC-1 gene (Sacchi *et al*., 2004) whereas only four alleles, showing a 1,500 bp repetitive region, were reported in sheep (Rasero *et al*., 2007). Accordingly, in SDS-PAGE, MUC-1 from sheep milk appears smaller in size than its caprine counterpart (Cebo and Martin, 2012).

The number of tandem repeats truly represents a matter of interspecies differences. In humans, it varies from 21 to 125 with 41 and 85 repeats being the most frequently encountered motif in the Northern European population. As a consequence, apparent molecular masses in SDS-PAGE for human MUC-1 range from 240 to 450 kDa whereas those for bovine MUC-1 are considerably lower (Gendler *et al*., 1990; Pallesen *et al*., 2001). Analysis of the polymorphism in six Italian breeds showed that the sheep repetitive region seemed to be less variable and smaller in size than the repetitive region of the goat.

Ruminants can be a useful model to study the mechanisms by which the variation in the repeat number and the extracellular domain size can modulate the effectiveness of MUC-1 as a cell-surface shield (Rasero *et al*., 2007). Remarkably, the polymorphic nature of the gene has been lost in mice and other rodents (Spicer *et al*., 1991).

MUC-1 is an extensively glycosylated mucin that may act as a decoy receptor for numerous pathogens invading epithelial tissues, including the mammary gland. Accordingly, numerous health-related (mostly, anti-infectious) benefits have been proposed for MUC-1 in milk (Schroten, 1998; Patton, 1999). Two studies investigated potential associations between the MUC-1 genetic polymorphism and important dairy traits, including milk fat or protein percentages or somatic cell counts in milk, an indirect measure of mastitis susceptibility in dairy cattle. Both studies concluded no association between the *MUC-1* genotype and somatic cell counts in milk. In addition, only weak associations between the number of tandem repeats in MUC-1 and milk protein and fat percentages were found in Holstein dairy cows (Hens *et al*., 1995; Sando *et al*., 2009).

15.5.1.2 Fatty Acid Synthase

Fatty Acid Synthase (FASN) is a complex homodimeric enzyme which catalyses *de novo* biosynthesis of long-chain fatty acids from acetyl-coenzyme A and malonyl-coenzyme A. *FASN* is therefore a strong candidate gene for fat content in milk. In addition, several studies reported quantitative trait loci on *Bos taurus* chromosome 19 (BTA19) where *FASN* maps (Roy *et al*., 2001; Boichard *et al*., 2003).

Two SNP were analysed in bovine *FASN* with regard to their associations with milk-fat content: they were located in exon 1 (g.763G>C) and in exon 34 (g.16009A>G) of the bovine *FASN* genomic sequence (GenBank # AF285607). The g.763G>C substitution alters a putative Sp1 transcription factor-binding site in the untranslated exon 1, whereas the g.16009A>G substitution in exon 34 generates a non-conservative substitution of a threonine with an alanine residue within the domain having enoyl and ketoacyl reductase

activities of FAS. Roy *et al*. (2006) reported significant frequency differences in Holstein cows with high and low breeding values for milk-fat content. Additional SNP in bovine *FASN* significantly associated with C14:0, C18:2cis9,12 and C18:1cis9 fatty acid levels in milk were reported recently (Morris *et al*., 2007; Schennink *et al*., 2009).

15.5.1.3 Butyrophilin

Butyrophilin (BTN1A1) is one of the most abundant proteins in the MFGM (Mather, 2000). Butyrophilin belongs to the B7/butyrophilin-like proteins, a subset of the immunoglobulin superfamily. The main features of butyrophilin are an extracellular part containing two Ig-like domains, a short transmembrane region and a long carboxy-terminal cytoplasmic domain called B30.2 domain. Interestingly, *BTN1A1* gene which is positioned within the cluster of *BTN* genes (seven genes including the two subfamilies, BTN2 and BTN3, arranged in pairs) is located close to the leukocyte antigen class I genes on human chromosome 6, thus linking butyrophilin to proteins involved in the immune response (Rhodes *et al*., 2001).

Butyrophilin plays a key role in the regulated secretion of milk lipids (Ogg *et al*., 2006). The prevailing model for milk lipid droplet formation in the MEC favours that adipophilin (ADRP or Perlipin*2/PLIN2*), at the surface of lipid droplets, mediates interactions with butyrophilin at the cytoplasmic face of the MEC apical membrane. These interactions, stabilised by XOR, initiate oligomerisation of butyrophilin leading to the final budding of lipid droplets and the release of fat globules in the luminal compartment (Heid and Keenan, 2005; MacManaman *et al*., 2007). However, this model is challenged by several studies suggesting that butyrophilin homophilic interactions solely orchestrate fat globule extrusion from MEC (Robenek *et al*., 2006a, b).

Previous studies have reported genetic variability within exon 8 of the butyrophilin gene in sheep (EMBL# **AY491475**), cattle (EMBL# **Z93323**) and buffalo (EMBL# **AY491471**). Two alleles (A, B) and three genotypes (AA, AB, BB) were reported for butyrophilin together with their frequencies in two ovine breeds (Karakul and Muzzafarnagari), Holstein-Friesian × Hariana cross-bred cattle and Murrah buffaloes (Bhattacharya *et al*., 2004, 2007). More recently, a novel SNP was reported in exon 5 of caprine *BTN1A1* (Qu *et al*., 2010). A single-nucleotide change (C to T) resulted in a missense mutation at position 377 of *BTN1A1* (leucine to phenylalanine substitution). This SNP was significantly associated with milk yield, as well as with milk fat and total solids percentage in Xinong Saanen dairy goat milk (Qu *et al*., 2010). Taken together, these studies provide substantial evidence for the existence of genetic polymorphism in *BTN1A1* gene in association with important dairy traits.

15.5.1.4 MFGE8

Milk-fat globule-epidermal growth factor (MFGE8) or lactadherin (LDH) is a major protein of the MFGM (Mather, 2000). The main feature of bovine LDH is the presence of two epidermal growth factor (EGF)-like domains in the N-terminal region of the protein with an arginine-glycine-aspartic acid (RGD) sequence in the second EGF-like domain and of two C terminal regions of about 150 amino acids called F5/8 type C or C1/C2-like domains also present in coagulation factors V and VIII (Hvarregaard *et al*., 1996). The C-terminal domain of the second F5/8 repeat has been shown to be responsible for membrane binding through a phosphatidylserine-binding motif (Shi and Gilbert, 2003; Shi *et al*., 2004). The RGD sequence is a cell-adhesion motif able to bind to $\alpha_v\beta_{3/5}$ integrins therefore mediating cell adhesion as well as cell transduction mechanisms (Taylor *et al*., 1997).

Multiple forms of LDH have been reported in the mammary gland and other tissues (Giuffrida *et al*., 1998; Häggqvist *et al*., 1999: Oshima *et al*., 1999). We have recently shown that LDH from goat milk appears as a single polypeptide chain contrary to its bovine counterpart which appears as two glycosylated polypeptides (Cebo *et al*., 2010). Furthermore, four polypeptides were identified among MFGM proteins from mares' milk, and LDH is expressed as two major protein variants in the camel (Cebo *et al*., 2012; Cebo

and Martin, 2012). Thus, although the occurrence of splicing variants has been reported for LDH, the molecular diversity of LDH across species is mostly explained by differential glycosylation of a single polypeptide backbone.

LDH is involved in a wide range of biological functions, including apoptosis, mammary gland involution after lactation and subsequent lactation (Hanayama and Nagata, 2005; Raymond *et al*., 2009). It is therefore expected that genetic polymorphism occurring within MFGE8 gene may affect lactation biology or milk composition in cattle.

The *LDH* gene (*MFGE8*) has been sequenced recently in the goat species (GenBank number GQ344829). Goat *MFGE8* consists of nine exons covering a 3,844 bp genomic sequence. A recent study (Qu *et al.*, 2010) investigated variations in the MFGE8 and its associations with growth traits and milk performance in the goat (*Capra hircus* taxon, Guanzhong dairy breed). Interestingly, the authors identified four loci in the goat *MFGE8* which were highly polymorphic. They were in the 5′-untranslated region (5′ UTR), the fourth intron, the seventh exon and the seventh intron. The g.14892T>C mutation resulted in a synonymous mutation, AAT (Asp)>AAC (Asp), located in the seventh exon of the gene. Research for codon preference in the goat *LDH* gene indicated that the preferred codon for Asp was AAC, the AAT sequence being the less preferred codon in this gene. This mutation, which changes from a low preference codon to a much higher preference one, may affect the expression level of the protein by modifying the mRNA stability.

Genetic polymorphism in the *MFGE8* gene has been shown recently to be associated with systemic lupus erythematosus (SLE) disease in humans. SNP were identified in codons 3 (arginine to serine substitution) and 76 (leucine to methionine substitution) of human *MFGE8*. The MFGE8 genotypic combination with arginine and methionine at positions 3 and 76 of the resulting protein, respectively, was identified as the most predisposing genotype to SLE in a case–control study (Hu *et al*., 2009). In addition, an A to G mutation located in intron 6 of human *MFGE8* caused the aberrant inclusion of a cryptic exon in the human transcript. This point mutation generates a premature termination codon resulting in a C-terminal truncated form of MFGE8 protein leading to the development of the disease in SLE patients (Yamaguchi *et al*., 2010).

15.5.1.5 ABCG2

ABCG2 (also known as BRCRP, breast cancer resistance protein) belongs to the ATP-binding cassette (ABC) family of transmembrane transporters. ABCG2 is strongly expressed during late pregnancy and lactation, and a role for secretion of riboflavin (Vitamin B2) into milk has been demonstrated for this transporter (van Herwaarden *et al*., 2007). In addition, ABCG2 has been identified in bovine MFGM (Reinhardt and Lippolis, 2006) as well as in the MFGM from goat and camel milk (Cebo *et al*., unpublished data). Another member of the ABC family, namely, ABCG1, has been recently identified in both basal and apical regions of mammary cells and in milk-fat globules (Mani *et al*., 2011). Because ABCG1 is involved in the active transport of sterols across the membrane, and may thus control cholesterol levels in milk, such a role for ABCG2 may not be excluded.

Segregating QTL for milk, fat and protein were reported on *Bos taurus* chromosome 6 (BTA6), and a 420 kb region near *ABCG2* was determined (Olsen *et al*., 2005). A missense mutation was finally identified within exon 14 of bovine *ABCG2*. The single-nucleotide change (A to C), induces a nonconservative tyrosine to serine (Y581S) substitution. $ABCG2^{A}$ (allele A of *ABCG2* capable of encoding a tyrosine residue), which was the most frequent allele in the Israeli Holstein population, was associated with an increased fat and protein concentration in milk (Cohen-Zinder *et al*., 2005). Interestingly, analysis of the corresponding *ABCG2* sequence in Indian breeds of cattle (*Bos indicus*) and buffalo (*Bubalus bubalis*) revealed fixed $ABCG2^{A}$ alleles, thus suggesting that $ABCG2^{A}$ is the ancestral allele and that the Y581S substitution ($ABCG2^{C}$ allele) occurred after the separation of *B. indicus* and *Bos taurus* lineages over 200,000 years ago (Ron *et al*., 2006; Tantia *et al*., 2006).

More recently, two novel SNP (45599A>C and 45610A>G) were reported in bovine *ABCG2*. These SNP, located within the seventh intron of *ABCG2*, were associated with milk yield and somatic cell scores (Yue *et al*., 2011).

15.5.1.6 FABP3

Fatty acid-binding proteins (FABP) constitute a family of small intracellular proteins (~15 kDa) which are involved in fatty acid transport from the plasma membrane to the sites of β-oxidation and triacylglycerol or phospholipid synthesis. To date, nine genes have been identified in mammals, and FABP were initially named according to their tissue of origin (Storch and Thumser, 2010).

The MFGM form of FABP has been shown to be heart-type FABP (FABP3) in the bovine (Mather, 2000) or, more recently, in the ovine (Pisanu *et al*., 2011) species. Because of its role in fatty acids transport and its expression in the lactating mammary gland, *FABP3* is a functional candidate gene for milk traits. Two SNP (one in exon 2 and the other one in intron 3) have been reported in ovine *FABP3* (Calvo *et al*., 2002). Both SNP were tested with regard to associations with milk yield, protein and fat contents. Results strongly suggest a role for *FABP3* genotype on milk-fat content (Calvo *et al*., 2004).

15.5.2 Genetic Polymorphisms Associated with Milk Proteins Encoding Genes Which Affect MFGM Composition

Besides the growing number of studies describing genetic variability in MFGM protein-encoding genes, it appears that the genetic polymorphism previously reported for non-MFGM milk proteins may affect both milk-fat structure (i.e. biophysical properties of milk-fat globules) together with MFGM composition.

Indeed, the extensive genetic polymorphism described in the goat at the *CSN1S1* locus (encoding the α_{s1}-casein protein in milk) is associated with strong differences in milk protein and milk-fat content (Grosclaude *et al*., 1994; Barbieri *et al*., 1995). Four levels of α_{s1}-casein synthesis have been described, ranging between 3.5 and 0 g/L for strong alleles (A, B and C) and null alleles (O), respectively (Martin *et al*., 2002). Moreover, in null animals for α_{s1}-casein, secretory pathways are severely affected. In the absence of α_{s1}-casein, an accumulation of immature proteins (mainly caseins) is observed, leading to a dramatic distension of the rough ER (Chanat *et al*., 1999). Since the first steps of milk-fat synthesis occur in the ER, this process might be affected by the general dysfunction of secretion pathways observed in α_{s1}-casein null goats. Accordingly, it was recently demonstrated that both milk-fat globule size and zeta potential are related to the α_{s1}-casein genotype. At mid-lactation, goats displaying strong genotypes for α_{s1}-casein (A/A goats) produced larger fat globules than goats with a null genotype at the *CSN1S1* locus (O/O goats). Moreover, dramatic differences were found with regard to MFGM composition (including both MFGM proteins and polar lipids) in the milk from goats with extreme genotype at the *CSN1S1* locus (Cebo *et al*., in press). Although the polar lipids composition of the MFGM have been shown to be modified by diet (Lopez *et al*., 2008), breed (Graves *et al*., 2007) or even lactation stage (Bitman and Wood, 1990), this is to our knowledge the first report of the impact of genetic polymorphism of milk proteins on the MFGM composition.

15.6 Linkage Between Casein Genes: The Haplotype Notion

One of the important features of milk proteins genetics, first highlighted by Grosclaude *et al*. (1965), is the tight linkage of the genes encoding the four caseins. This situation has been confirmed 25 years later (Ferretti *et al*., 1990; Threadgill and Womack, 1990). Casein genes are organised as a cluster spanning a ca. 250 kb DNA fragment located on chromosome 6 in cattle and goats. The haplotype notion, coined in human histocompatibility studies, has been introduced to designate a combination of alleles of closely linked genes (Ng-Kwai-Hang and Grosclaude, 1992). The non-independent inheritance of casein alleles in popu-

lations is an example of "linkage disequilibrium" (non-random association of alleles). The strongest linkage occurs between alleles at *CSN1S1* and *CSN2* loci which are convergently transcribed and between 10 and 20 kb apart, across species. Even though recombination may occur at a very low frequency between so close loci, the casein cluster is considered as a unit of inheritance: a haplotype. This haplotype notion is useful to improve the estimation of the relationship between casein variants and milk production traits. Considering the haplotype instead of single alleles at the four casein loci was first suggested by Grosclaude *et al*. (1979) in cattle and further developed in the same species (Ikonen *et al*., 2001; Boettcher *et al*., 2004; Caroli and Erhardt, 2004).

15.6.1 Relationship Between Casein Variants and Milk Production Traits

Practical applications emerging from the study of genetic polymorphism of milk proteins have been extensively reviewed by Ng-Kwai-Hang and Grosclaude (2003) in the third edition of *Advanced Dairy Chemistry-1: Proteins*. Using milk protein genes as genetic markers for increasing milk production, improving production-related traits and altering milk composition remain a matter of interest. However, a new technology called genomic selection (Meuwissen *et al.*, (2001) is revolutionising dairy cattle breeding. Genomic selection refers to selection decisions based on genomic breeding values (GEBV) which are calculated as the sum of the effects of dense genetic markers (SNPs), or haplotypes of these markers, across the entire genome, thereby potentially capturing all the quantitative trait loci (QTL) that contribute to variation in a trait (Hayes *et al*., 2009). This approach which is a variant of marker-assisted selection has become feasible, thanks to the large number of SNPs discovered by genome sequencing and new methods to efficiently genotype large number of SNP (Goddard and Hayes, 2009).

However, there are still many reports that relate genetic polymorphism of milk proteins to milk-fat or milk protein content, and the haplotype concept has been refined. A high-resolution SNP map of the bovine 2009 region was recently constructed by Nilsen *et al.* (2009) to study associations with milk traits in Norwegian Red cattle (NRC). Consistent with the structure of the casein cluster they suggested to separate the casein cluster, into two haplotype blocks, one consisting of *CSN1S1*, *CSN2* and *CSN1S2* and the other consisting of *CSN3*. Associations both with protein and milk yield were found highly significant within the *CSN1S1-CSN2-CSN1S2* haplotype block. In contrast, no significant association was found within the *CSN3* block. The authors pointed towards *CSN2* and *CSN1S2* as the most likely genes harbouring the underlying causative DNA variation. The most significant results involved the *CSN2_67* SNP, a transversion C > A in codon 67 that results in Pro to His exchange, with C being consistently associated with greater protein and milk yield. More recently, the same team (Sodeland *et al.*, 2011), using high coverage re-sequencing, enabled molecular characterisation of a long-range haplotype encompassing genes *CSN2 and CSN1S2* from a Swedish Holstein-Friesian bull introduced into the NRC population. Haplotype analysis of a large number of descendants from this bull indicated that the haplotype which was not markedly disrupted by recombination in this region was associated both with increased milk protein content (SNPs possibly affecting transcription and/or translation of *CSN1S2*) and increased susceptibility to mastitis (polymorphisms close to a cluster of genes encoding CXC chemokines).

In a recent investigation of the Dutch Holstein-Friesian population (Heck *et al*., 2009), it has been shown that genetic variants and casein haplotypes have a major impact on the protein composition of milk and explain a considerable part of the genetic variation in milk protein composition. It was concluded from this study that selection for both the B allele at the *LGB* locus (*LGB*B*) and the *CSN2*A2-CSN3*B* haplotype will result in cows that produce milk more suitable for cheese production. A linkage study performed to screen the whole bovine genome identified ten chromosomal regions (QTL) affect-

ing milk protein composition (casein, whey protein and specific protein content). Regions on *Bos taurus* autosomes (BTA) 6, 11 and 14 showed the largest effect on milk protein composition, and some QTL could partially be explained by polymorphisms in milk protein genes (Schopen *et al.*, 2009). Using a whole-genome association study, three genomic regions associated with major effects on milk protein composition or protein percentage were confirmed on BTA6, BTA11, and BTA14 in addition to several regions with smaller effects involved in the regulation of milk protein composition (Schopen *et al*., 2011).

Taken together, these results strongly suggest that there are opportunities to increase the casein index which is a desirable breeding goal for cheesemaking properties and cheese production and that it would be advisable to combine information on polymorphism of known genes (casein cluster) with genomic selection (anonymous markers).

Several association studies have analysed the effects of the polymorphism of casein genes on dairy performance and milk quality in different goat breeds (Grosclaude *et al*., 1994; Grosclaude and Martin, 1997; Ådnøy *et al*., 2003; Hayes *et al*., 2006; Chilliard *et al*., 2006; Chiatti *et al*., 2007). They revealed that polymorphisms at the *CSN1S1* locus have significant effects on casein content, total protein content, fat content and technological properties of milk. It has also been reported that κ-casein (CSN3) variants have a significant influence on milk production traits (Angulo *et al*., 1994; Chiatti *et al*., 2007; Caravaca *et al*., 2009).

Among these studies, one of the most genetically detailed was performed on Norwegian goats by Hayes *et al*. (2006) who identified and used 39 polymorphisms (SNPs) within the casein genes to assess the effect of these haplotypes on milk production traits. Most of these SNPs are located in the promoter regions of the genes (particularly for *CSN3*). The numbers of unique haplotypes found in a large population of Norwegian bucks in each locus were 10, 6, 4 and 8 for *CSN1S1*, *CSN2*, *CSN1S2* and *CSN3*, respectively. The effects of the *CSN1S1* haplotypes on protein percentage and fat amount were significant, as were the effects of *CSN3* haplotypes on fat percentage and protein percentage. A deletion in exon 12 of *CSN1S1*, so far reported only in the Norwegian goat population and at a high frequency (0.73), explained the effects of *CSN1S1* haplotypes on fat amount, but not protein percentage. Investigation of linkage disequilibrium between all possible pairs of SNPs revealed higher levels of linkage disequilibrium for SNP pairs within casein loci than for SNP pairs between casein loci, likely reflecting low levels of intragenic recombination. Further, they provide evidences for a site of preferential recombination between *CSN2* and *CSN1S2*. Casein haplotypes were found to have large effects on production traits, and the possibility to use haplotypes associated with the increase in fat and protein percentages, in haplotype-assisted selection, would have potential. In a second study, in which both genotype and phenotype information on milk-producing goats were recorded, casein SNP dominance and additive effects were investigated (Dagnachew *et al.*, 2011). Unlike in the previous study, the deletion occurring in exon 12 appeared to be significantly associated with protein and fat contents and with milk taste. Similar results have been reported previously by Grosclaude *et al.* (1994) and Grosclaude and Martin (1997) who underlined the unexpected effect of defective alleles at the *CSN1S1* locus (alleles *E* and *F*) on fat content, correlated with a dysfunction in secretory mechanisms triggered by the accumulation of caseins in the endoplasmic reticulum of the MEC (Chanat *et al*., 1999). They also reported that the polymorphism at the *CSN1S1* locus in French dairy flocks (Alpine and Saanen breeds) has significant effect on the diameter and calcium content of casein micelles which were both lower in AA milks. Moreover, in traditional cheesemaking of Pélardon des Cévennes-type cheeses, the goat flavour tended to be less pronounced in AA milks. The "goaty" flavour in cheese was partly due to lipolysis occurring in milk before clotting. On the other hand, they did not observe any association between genetic variants of α_{s1}-casein and milk yield.

15.6.2 Genetic Variants of Milk Proteins for Population and Phylogeny Studies

Haplotypes at the casein cluster as well as genetic variants of milk proteins were also intensively used for characterising breeds and as markers for population and phylogeny studies, establishing geographical diversity (see review by Ng-Kwai-Hang and Grosclaude, 2003). Jann *et al*. (2004) investigated the diversity of the casein locus in the context of the origin and phylogeny of taurine cattle, including variants which had not been yet the subject of phylogeny studies. The 19 alleles at the 5-linked loci (including one SNP in the promoter region of *CSN1S1*) were combined in 83 haplotypes. Genotyping of 30 cattle breeds from four continents revealed that casein haplotype frequencies are geographically distributed and defined mainly by frequencies of alleles at the *CSN1S1* and *CSN3* loci. The genetic diversity within *Bos taurus* breeds in Europe was found to decrease significantly from the south to the north and from the east to the west. Such geographic patterns of cattle genetic variation at the casein gene cluster may be a result of the domestication process of modern cattle as well as geographically differentiated natural or artificial selection. The comparison of African *Bos taurus* and *Bos indicus* breeds allowed the identification of several *B. indicus*-specific haplotypes not found in pure taurine breeds. The occurrence of such haplotypes in southern European breeds also suggests that an introgression of indicine genes into taurine breeds could have contributed to the distribution of the genetic variation observed. Such a hypothesis is substantiated by several studies performed on Portuguese cattle breeds (Beja-Pereira *et al*., 2002), on the Original Pinzgauer red and white cattle breed, from the Pinzgau region of the federal state of Salzburg in Austria (Caroli *et al*., 2010) and on 26 breeds from ten countries spanning three continents (Ibeagha-Awemu *et al*., 2007). It was found in the first two studies that the CA2 haplotype (abbreviation of *CSN1S1*C-CSN2*A2*) which is the most frequent haplotype in zebu breeds (Mahé *et al*., 1999) also predominates in several European cattle breeds, including Portuguese breeds and the Austrian breed. Ibeagha-Awemu *et al*. (2007) demonstrated, using alleles *CSN1S2*B* and *CSN3*H* as markers, that zebu-specific attributes may be more widely distributed in European cattle breeds than expected from previous data.

Sacchi *et al*. (2005) investigated the genetic structure of the casein gene cluster in five Italian goat breeds to evaluate the haplotype variability within and among populations. Goats from Vallesana, Roccaverano, Jonica, Garganica and Maltese breeds were genotyped at the four casein loci (*CSN1S1*, *CSN2*, *CSN1S2* and *CSN3*) using genomic techniques and milk protein analysis. Allele and haplotype distributions indicated considerable differences across breeds. *CSN2* appeared to be monomorphic for the A allele. The haplotype *CSN1S1*F-CSN1S2*F-CSN3*D* occurred in all breeds and was the most common haplotype in the Southern breeds. A high frequency of *CSN1S1*0-CSN1S2*C-CSN3*A* haplotype was found in the Vallesana population (0.162). Principal component analysis clearly separated the Northern and Southern breeds. The authors suggest that the variability of the caprine casein loci and variety of resulting haplotypes should be exploited in the future using specific breeding programmes aiming to preserve biodiversity and to select goat genetic lines for specific protein production. Interestingly, a Maltese goat heterozygous for *CSN2*0* was homozygous for the F allele at *CSN1S1* locus. This indicates the occurrence of a *CSN1S1*F-CSN2*0* haplotype, which is associated with a very low casein content even if linked to a strong *CSN1S2* allele.

A similar investigation was carried out by Caroli *et al*. (2006) with local Lombardy breeds and confirmed that the casein-haplotype structure is highly different among breeds. Combining haplotype with the molecular knowledge of each locus, it was postulated that the ancestral haplotype might be *CSN1S1*B-CSN2*A-CSN1S2*A-CSN3*B*. A casein cluster evolutionary model considering the whole casein haplotype was proposed, and strong evidence of recombination events, not only among but also within casein genes, was found. This is consistent with the interallelic recombination hypothesis put forward

to account for the occurrence of the M α_{s1}-casein variant in the goat species and the postulated existence of two ancestral allelic lineages at the *CSN1S1* locus (Bevilacqua *et al*., 2002).

In addition to assess the phylogenetic relationships, genetic polymorphisms of milk proteins have also been used to investigate the genetic diversity within and between populations to establish geographical diversity. Tadlaoui Ouafi *et al.* (2002) have shown that *CSN1S1* alleles associated with a high expression level (mainly A and B) are predominant in Moroccan goat breeds (74% and 94% in Draa and Noire-Rahalli, respectively), whereas allele E, which is rather frequent in European goat breeds, is rare (2–3%).

To characterise the diversity within the four casein genes in two geographically distant goat populations, the Sicilian Girgentana breed and the Norwegian goat breed, goats were haplotyped, based on 22 SNPs and one deletion (Finocchiaro *et al.*, 2008). The SNP haplotype frequencies for the four casein genes were calculated, and despite the large geographical distance and phenotypic divergence between these two breeds, a proportion of casein loci haplotypes were found to be identical between both Norwegian and Girgentana goats. The level of linkage disequilibrium between the casein genes was less in the Girgentana population than in the Norwegian population.

Milk protein polymorphism was also investigated in nine Indian goat breeds/genetic groups from varied agro-climatic zones to analyse the genetic structure of the casein cluster and milk protein diversity at six milk protein loci (Rout *et al.*, 2010). Frequencies of the A allele at the *CSN1S1* locus varied from 0.45 to 0.77, and a total of 16 casein haplotypes were observed in seven breeds. The distribution of casein haplotypes was specific to breed and geographical regions. The average number of alleles was lowest in Ganjam (1.66 ± 0.81) and highest in Sirohi goats (2.50 ± 1.05). Expected heterozygosity at six different loci (caseins α-lactalbumin and β-lactoglobulin) demonstrated genetic diversity and breed fragmentation. There was about 17% variability due to differences between breeds; neighbour-joining tree built using Nei's distance, indicating a strong subdivision.

15.7 Milk Proteins Polymorphism and Human Nutrition

Our nutritional perception of milk has grown substantially from a time when it was seen purely as an excellent source of protein and calcium. Milk proteins provide the suckling neonate with a source of amino acids, highly bioavailable calcium, but also potentially health-promoting bioactive peptides (i.e. antimicrobial, antihypertensive, immune modulating). Thus, over the last decade, attention has shifted from the contribution of milk proteins to human nutrition in terms of essential amino acids (summarized in Table 15.17) to more specific human health issues, not only restricted to the neonate, such as the presence of bioactive peptides encrypted in milk proteins and allergy to milk proteins. Biological activities of peptides released from milk protein digestion are directly impacted by amino acid substitutions or internal deletions arising from gene mutations. The existence of defective alleles associated with a reduced content of different caseins is also of interest for the production of hypoallergenic milk with a low casein content. Since PTMs such as phosphorylation seem to reduce the allergenicity of cow caseins in children with selective allergy to goat and sheep milk (Cases *et al*., 2011), mutations impacting the phosphorylation level of caseins might be a selection goal.

15.7.1 Bioactive Peptides

Milk proteins are essentially the only source of amino acids for the newborn mammal and a common source of proteins for adults, but their larger physiological significance has started to be acknowledged only by the end of the twentieth century. Fiat and Jolles (1989) reviewed the structural and physiological aspects of caseins and the presence of potentially bioactive peptides, but the amount of information concerning the biological effect of these compounds in vivo was still scarce at the time. At present, milk proteins are regarded as a major source of bioactive peptides, and the number of biologically active sequences as well as

Table 15.17 Supply of essential amino acids (g/100 mL) from bovine, caprine and ovine milka and percentage of Recommended adult Daily dietary Allowances (RDA) fulfilled (g/day)

	Cow milk		Goat milk		Sheep milk		
	Supply	% RDA	Supply	% RDA	Supply	% RDA	RDA (g/day)
Tryptophan	0.04	*8*	0.04	*8*	0.08	*16*	**0.50**
Threonine	0.16	*16*	0.16	*16*	0.28	*28*	**1.00**
Isoleucine	0.2	*14.2*	0.22	*15.8*	0.34	*24.2*	**1.40**
Leucine	0.32	*14.6*	0.32	*14.6*	0.6	*27.2*	**2.20**
Lysine	0.26	*16.2*	0.3	*18.8*	0.52	*32.4*	**1.60**
Methionine	0.08	*3.6*	0.08	*3.6*	0.16	*7.2*	**2.20**
Cystine	0.04	–	0.04	–	0.04	–	–
Phenylalanine	0.16	*7.2*	0.16	*7.2*	0.28	*12.8*	**2.20**
Tyrosine	0.14	–	0.18	–	0.28	–	–
Valine	0.22	*13.8*	0.24	*15*	0.44	*27.6*	**1.60**

Adapted from Haenlein (2001)

[a]Average composition of milk: cow, 12.01% total solids, 3.29% protein; goat: 12.97% total solids, 3.56% protein; sheep: 19.30% total solids, 5.98% protein

the spectrum of activities is constantly increasing. Bioactive peptides are specific protein fragments that have a positive effect on physiological functions or conditions and might ultimately influence health (Kitts and Weiler, 2003). They are said to be encrypted (latent) within the primary structure or parent proteins and need to be released upon proteolysis to exert a physiological effect. Their activity is based on their inherent amino acid sequence. The size of active peptides may vary from 2 to 20 amino acid residues, and many peptides are known to have multifunctional properties (Meisel and FitzGerald, 2003). The origins and biological effects of such peptides have been reviewed by several authors (Shah, 2000; Clare and Swaisgood, 2000; Hayes *et al*., 2007; Korhonen, 2009).

Despite the importance of the amino acid sequence of such peptides, little or no attention was paid to the genetic polymorphisms of milk proteins until the outbreak in the late 1990s of the "A2 milk case", as critically reviewed by Truswell (2005). Briefly, the single amino acid substitution P67H in bovine β-CN A1, compared to A2, allows the cleavage by pepsin, leucine aminopeptidase and elastase, releasing the opioid peptide known as β-casomorphin-7 (BCM7), corresponding to the sequence 60–66 of bovine β-CN (Tyr-Pro-Phe-Pro-Gly-Pro-Ile). In turn, the possible presence of BCM7 in dairy products was allegedly associated with an array of diseases, ranging from type-I diabetes mellitus, to atherosclerosis and coronary-heart diseases, and later to central nervous system disorders, such as autism. A recent report by the European Food Safety Authority (EFSA, 2009) extensively revises the possible implication of BCM7 and other β-casomorphins in the development of human infirmity and concluded that "a cause-effect relationship between the oral intake of BCM7 or related peptides and aetiology or course of any suggested non-communicable diseases cannot be established". In this respect, caution should be taken when claiming the health effects of milk bioactive peptides, be it beneficial or detrimental. Nevertheless, the possible presence of bioactive peptides encrypted in different milk protein variants was definitely acknowledged and widened the horizons of this research field.

Two interesting examples on this subject were reviewed by Caroli *et al*. (2009). In the first one, Weimann *et al*. (2009) compared AA sequences of several bovine κ-CN variants and predicted the sequence of four different antihypertensive peptides (ASP, variant B and C; VSP, variant F1; AHHP, variant C; ACHP, variant G2). Such peptides were synthesised and their ACE inhibitory potency was demonstrated in vitro. In the second one, Tulipano *et al*. (2010) investigated the effects of four chemically synthesised peptides, encrypted in bovine caseins (β-and α_{s2}-casein)

and corresponding to genetic variants showing one amino acid change in their sequence, on osteoblast mineralisation in vitro. Results suggested that distinct peptides in protein hydrolysates may differentially affect calcium deposition in the extracellular matrix and that the genetic variation within the considered sequences may profoundly impact their biological activities. More recently, Norberg *et al.* (2011) assayed the antimicrobial potency of synthetic homologues of two previously reported bovine α_{s1}-casein peptides (caseicin A, IKHQGLPQE; caseicin B, VLNENLLR) against many Gram-negative pathogens and *Staphylococcus aureus*. The effect of single amino acid substitutions was compared to the original sequences, and it appeared that the importance of specific residues within the caseicin peptides is dependent on the strain being targeted.

Taken together, these findings suggest that milk protein polymorphisms and amino acid substitutions naturally occurring not only in the cattle but also in the caprine and ovine species, greatly increase the possibility of discovering new sequences of biologically active peptides.

15.7.2 Milk Allergy

As reviewed by Crittenden and Bennett (2005), in spite of the substantial differences in the types and sequence of proteins that are present in ruminant and human milk, in most people the immune system is able to recognise these proteins as harmless and tolerate them. In some individuals, however, the immune system becomes sensitised to the milk proteins and mounts a damaging inflammatory response, for reasons that are not still completely understood. In a recent work, Wal (2004) stated that most of milk proteins may be regarded at as potential allergens, as several epitopes, both conformational and linear, have been described so far.

Under this viewpoint, amino acid substitutions could occur in the areas of highly conserved milk protein epitopes described so far, and this could limit their allergenicity. Thus, the selection of animals on specific qualitative polymorphisms could be a way to theoretically reduce the immunogenicity of milk proteins. Preliminary works by Chessa *et al.* (2008) in silico, suggested that genetic variability could impact the structure of IgE-binding epitopes structure of milk proteins. Moreover, it can be argued that mutations affecting the phosphorylation of caseins could affect the immunogenicity of proteins, as observed in certain internally deleted variants or when the phosphorylation consensus sequence is modified. This hypothesis was tested by Bernard *et al.* (2000), who measured in vitro the specific IgE response to naturally occurring common variants of β- and α_{s2}-casein, both in the native and dephosphorylated forms, and a purified α_{s2}-casein variant D, lacking one major phosphorylation. Results showed that the IgE response to caseins was significantly reduced by modifying or eliminating the major phosphorylation site.

An even more interesting strategy, however, is offered by the quantitative polymorphisms described in a previous section of this chapter, namely, by the presence of "null" alleles, as observed in the goat species. In particular, selected dairy animals can be considered as natural knockout for major milk allergens, α_{s1}-, β- and α_{s2}-casein. Only little attention has been paid, to date, to the possibility of breeding dairy animals to target milk protein allergy patients. Bevilacqua *et al.* (2001) compared the effect of diets containing either cow milk proteins or goat milk proteins with high and low α_{s1}-casein content, on a guinea pig model, and analysed the sensitisation of animals against BLG. Interestingly, it was observed that animals fed the goat milk proteins diet low in α_{s1}-casein had significantly low anti-goat BLG IgG1 antibodies. The authors suggested that the high genetic polymorphism of goat milk proteins could account for different responses in terms of allergy to cow and goat milk. Marletta *et al.* (2004) tested the in vitro allergenicity of milk from goats: homozygous *N/N* at the *CSN1S2* locus (*N* being any allele except *O*), homozygous *O/O* or heterozygous *N/O* goats. The absence or the reduction of α_{s2}-CN in goat milk decreased but did not erase the allergenic potency of the casein fraction. More recently,

Ballabio *et al*. (2011) assessed the immunogenicity of milk from goats with different *CSN1S1* genotypes, both in vitro (SDS-PAGE and immunoblotting with sera from milk allergic patients) and in vivo (skin prick tests). A lower reaction was observed with milk samples from goats homozygous O1/O1 and O1/F at the *CSN1S1* locus. Moreover, skin prick tests, carried out on six allergic children, were negative to O1/O1 goat milk. The authors concluded that milk from animals carrying *CSN1S1* "weak" or "null" alleles should be tested in the preparation of modified formulas for selected groups of allergic patients.

15.8 Concluding Remarks

Advances made over the past decade in areas such as molecular biology, genomics and proteomics mainly due to major technological progress have greatly accelerated the process of acquiring knowledge. In particular, the primary structure of most milk proteins and the genomic organisation of the relevant genes have been elucidated providing access to a better understanding of the mechanisms regulating gene expression. This information has also opened many avenues of research and permits the development of more powerful selection tools. However, most traits of economic importance in livestock are either quantitative or complex. Despite considerable efforts, there have been only rare successes in identifying the causal polymorphisms responsible for variation in these traits. Genomic tools, such as SNP chips, have been used to identify genes involved in the expression of many traits, including dairy traits and to select genetically desirable livestock. This has led to the discovery of the causal mutations for several single-gene traits but not for complex traits. Genome-wide panels of SNPs have led to a new method of selection called "genomic selection" in which dense SNP genotypes covering the whole genome are used to predict the breeding value. Even though this approach is expected to double the rate of genetic improvement per year in many livestock systems (Goddard and Hayes, 2009), molecular information on loci showing a major effect has to be included.

Variation in milk protein composition is due primarily to mechanisms other than protein sequence polymorphisms. Mechanisms, such as transcriptional and translational regulation of milk protein-encoding genes, contribute to milk composition variation. Non-coding regions of the genome, particularly those with putative regulatory function, have been investigated intensively, thanks to an easier access to NGS technologies, and will be still further explored for mutations (SNP, indels) in the future. During very recent years, the shift from coding to non-coding sequences is probably the most striking evolution recorded as far as milk protein polymorphism analysis is concerned. Expression takes more and more importance. Mutations altering protein structure and expression, and therefore milk composition and properties, have been found in coding sequences, promoters, 3′- and 5′-untranslated regions and in intragenic as well as in intergenic regions. Novel mechanisms of regulation such as those found at the RNA level controlling traductibility and stability have been described (e.g. miRNA regulation of human lactoferrin expression).

Interestingly, quite the same events have been found in different species: insertion of repetitive elements (LINE) in the last non-coding exon of *CSN1S1* in goat and cattle and mutations impacting the splicing process more or less deeply (exon skipping, cryptic splice site usage). Differential splicing, affecting particularly genes whose structure is highly fragmented, or the occurrence of nonsense mutations (premature stop codons) was found to be a factor of heterogeneity and structural diversity as well as quantitative, with sometimes unexpected dramatic consequences.

These mutational events consequently affect either the coding message, the mRNA stability or both, leading to truncated protein products in weak amount or even their absence (null alleles). The occurrence of premature stop codons, due to single-nucleotide deletion (*CSN1S1* and *CSN2* in goat) or to SNP, triggers cellular response (mRNA decay) ensuring improper mRNAs to be degraded. Alleles *CSN1S1*G*, in goat, and *CSN1S1*A*, in cattle, both give rise to a messenger lacking exon

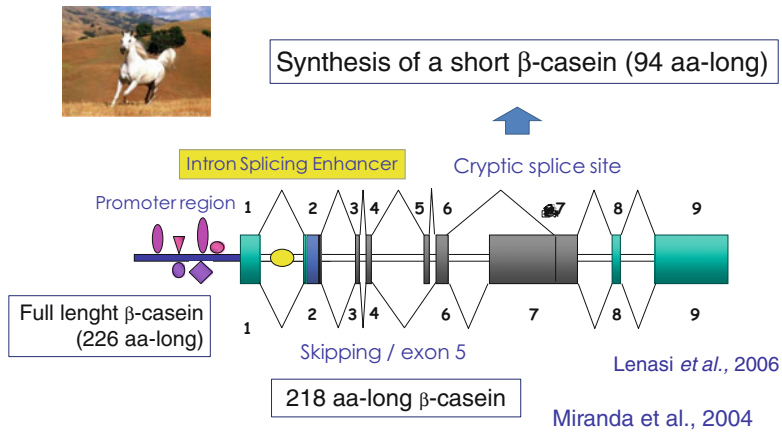

Fig. 15.4 A schematic representation of the multiple splicing patterns of the horse β-casein encoding gene (*CSN2*). A mutation in a splicing enhancer (*yellow circle*) within the first intron of the horse β-casein gene induces the usage of a cryptic splice site in exon 7 leading to the synthesis of a short β-casein (94 aa long). It also induces a casual (stochastic) skipping of exon 5 (a weak exon) responsible for an internal deletion of eight amino acid residues

4 which is skipped consecutively to a SNP at position +1 or +6 in the 5′ consensus splicing donor sequence of intron 4, respectively, resulting in the upstream exon skipping along the course of primary transcripts processing, with reduced α_{s1}-casein expression.

Another situation exemplifying the importance of the splicing process is provided in the equine species by a *cis*-element in intron 1 (intronic splicing enhancer 1, ISE1) of the gene encoding β-casein. This splicing enhancer increases the inclusion of all weak exons in its mRNA and is responsible for a cryptic splice site usage (Fig. 15.4) leading to the loss of more than a half of the coding sequence (Lenasi *et al.*, 2006). Thus, by producing different mRNA forms from a single gene, such mechanisms contribute to milk proteome diversity and complexity.

Nowadays, tools are available and milk protein polymorphisms are well documented, at least for the dairy ruminant species. It remains to gain further insights into the variability of proteins from the MFGM as well as quantitatively minor whey proteins and to acquire knowledge on the consequences of described polymorphisms on biological functions of milk proteins before initiating selection for milk production differentiated according to the targeted goals.

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Nutritional Quality of Milk Proteins

16

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16.1 Introduction

Milk has been a subject of nutrition research for many years because it represents a major source of nutrients, especially of protein, for populations worldwide. Generally, the protein content of cows' milk varies among cattle populations, between breeds and between individuals, with genetics accounting for most of the differences as protein content is more highly heritable than other components. Apart from genetic factors, natural variations in milk protein content are due to the stage of lactation, milk production, the cow's age, number of lactations and mastitic infections. Variations of milk protein content are also related to the cow's feeding system parameters, such as energy intake (positively), lipid supplementation (negatively), protein levels and their source and additional amino acid (AA) supplementation (in general positively). Many attempts have been made to increase protein yield and change the relative synthesis of the individual proteins. These results would improve both the technological and nutritional properties of milk. However, dietary manipulation of the amount and the composition of milk proteins is not easily feasible. Few studies have demonstrated significant changes in milk protein composition by balancing AAs in the dairy cows' diet via supplementation with different proteins or rumen-protected individual AAs, especially lysine and methionine.

Taking into account all of the aspects mentioned above, bovine milk contains about 32 g protein/L, caseins (~80%) and whey proteins (~20%) representing the two main fractions. Both protein groups have been recognised as fundamental in maintaining health and well-being, especially for the newborn, and as important components of a balanced diet.

In general, a lack of protein will have a negative impact, especially when protein requirements are higher (growth, pregnancy, feeding, ageing, illness), and, for this reason, it becomes indispensable that the daily recommended protein intake be assured. Clearly, this would be least problematic for populations in industrialised countries. There are new aspects, however, of increasing concern for consumers when choosing among different sources of dietary protein, such as cost and the resources needed and the environmental impact associated with their production.

Multidisciplinary research programmes are increasingly dedicated to investigating policy options for defining more sustainable diets through a reduction of environmental pressures related to protein production. Scoring food models based on the nutrient density and taking ecological, economic and cultural aspects into consideration are

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approaches being applied to studies of sustainable and affordable nutrition. Among these models, the Nutrient Rich Foods Index was used in conjunction with a food price database to rank different food groups (Drewnowski, 2010). Using this approach, milk proved to have the lowest overall nutrient-to-price ratio and to be the lowest-cost source of calcium, whereas milk products, along with eggs and legumes, were the lowest-cost sources of protein.

16.2 The Nutritional Quality of Dietary Proteins

The nutritional quality of a dietary protein is the expression of its bioavailability and metabolic utilisation. In other words, it represents the capacity of a food protein to achieve defined nutritional and metabolic actions related to a specified requirement. The evaluation of protein quality allows one to establish a ranking of food sources on the basis of their potential nutritive value and to predict the contribution of a protein to satisfy the nitrogen (N) and AA requirements for growth and maintenance of the human body. The classic approach to protein quality evaluation involves the coverage of human requirements to be assessed for each food source separately. Although such a concept is useful in terms of protein classification or measuring the effect of processing on the nutritional quality of single proteins, no prediction of diet quality can be made on this basis. However, the evaluation of dietary protein quality should involve multiparametric studies taking into account both the composition of the diet and the effect of the interaction of proteins with other components. At present, protein quality is determined from growth measurements, protein and N digestibility data, N balance, N retention or protein turnover at a whole-body level or in specific tissue protein pools (Tomé, 2010).

16.2.1 Protein Requirements

The understanding of proteins' actions has improved through the years, and evidence of proteins' complex roles in the regulation of various functions other than maintaining body protein mass has appeared in literature. Among several metabolic actions, Millward *et al*. (2008) reported the regulation of body composition, bone health, gastrointestinal function and bacterial flora, glucose homeostasis, cell signalling and satiety. Meanwhile, it is well known that protein deficiency depresses immune function and defence against diseases. A deficiency of protein reduces the plasma availability of most AAs, especially glutamine, arginine, methionine, tryptophan and cysteine, whose roles in enhancing immune function have been well established (Li *et al*., 2007; Tan *et al*., 2009; Wu, 2009), whereas high-protein diets may increase the acidity of the urine leading to an increased loss of minerals, in particular magnesium and calcium (Remer, 2001).

Currently, the N balance method, which consists of measuring the difference between protein intake and loss, is the favoured approach for estimating protein requirements in humans. A detailed definition of protein requirements was provided by the FAO/WHO Ad Hoc Expert Committee (1973), which defines it as 'the lowest level of dietary protein intake that will balance the losses of N from the body, and thus maintain the body protein mass, in persons at energy balance with modest levels of physical activity, plus, in children or pregnant/lactating women, the needs associated with the deposition of tissues or the secretion of milk at rates consistent with good health'. The study of Rand and Young (1999), consisting of a meta-analysis of previously published data for the estimation of protein requirements in healthy adults, is the basis for the most recent international recommendations. The FAO/WHO/UNU Expert Consultation (2007) proposed an estimated 'average requirement' of 0.66 g protein/kg body weight/day to achieve a zero N balance in healthy adults. Taking into account the interindividual variability, a 'safe level of intake' of 0.83 g protein/kg/day is expected to satisfy the requirements of most (97.5%) of the healthy population. Such requirements must be integrated with additional amounts of protein for children and pregnant/lactating women. In fact, the dietary requirement is the amount of protein that must be supplied in the diet to satisfy the metabolic demand and should, therefore, take into

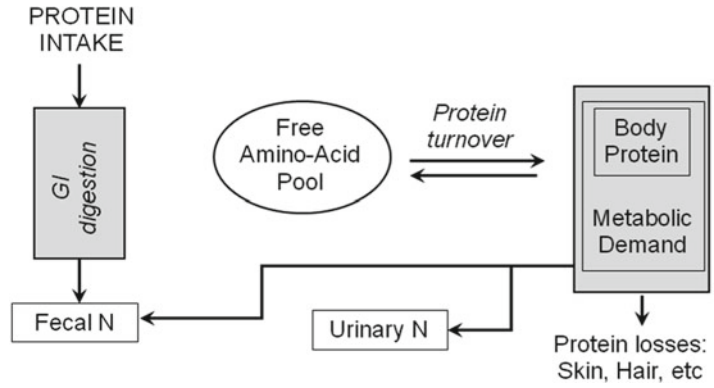

Fig. 16.1 Schematic representation of the metabolic demands for amino acids

account such factors as biological value and digestibility of the protein. The representation of the metabolic demands in Fig. 16.1 explains why balance methods are extremely difficult to apply and thus are imprecise. Milk proteins play a major role in ensuring that this intake is met, and by way of example, a survey by the French Agency for Food Safety (AFSSA, 2007) indicated that milk and milk products supplied 20.6% and 17.2% of dietary proteins for children (4–14 years) and adults, respectively. Despite the many unanswered nutritional issues, milk proteins provide a wide range of important functional and biological properties that are continuously being discovered and for which scientific evidence is accumulating.

16.2.2 Amino Acid Requirements

Although over 300 AAs exist in nature, only 20 of them, all in the L configuration, are used as structural units for building proteins. Nevertheless, it has been demonstrated that nonprotein α-AAs (like ornithine, citrulline, homocysteine), as well as non-α AAs (like taurine or β-alanine), play important metabolic roles (Perta-Kajan *et al.*, 2007; Manna *et al.*, 2009).

Amino acids are classified as nutritionally indispensable (IAA), dispensable (DAA) or conditionally indispensable (CIAA) for humans (Table 16.1). Indispensable AAs, all present in milk proteins, are defined as either those AAs whose carbon skeletons cannot be synthesised or those that are inadequately synthesised by the body and therefore must be provided by the diet. Nutritionally, DAAs can be synthesised in adequate amounts by the body in any situation, whereas CIAAs are those that can normally be synthesised in adequate amounts by the organism but that must be provided by the diet to meet optimal needs under particular conditions when their utilisation is more rapid than their synthesis. This classification is of the utmost importance because the bioavailability level of IAAs in a protein source is the key factor in determining its nutritional value. The mixture of dispensable AAs and CIAAs as supplied by adequate intake of food proteins will assure that both the N and specific AA needs are met.

Besides their fundamental function as building blocks for proteins, AAs also act as cell signalling molecules and regulate numerous important metabolic pathways necessary for maintenance, growth, reproduction and immunity. Furthermore, they are key precursors of hormones and other substances of great biological importance, like polyamines, glutathione, taurine, nitric oxide and serotonin. Tyrosine, for example, is the precursor for the synthesis of epinephrine, norepinephrine, dopamine and thyroid hormones, whereas glutamine and leucine increase insulin release from pancreatic β-cells (Newsholme *et al.*, 2005). An excess or deficiency of even one single AA can disturb whole-body homeostasis, can provoke problems of growth and development and may in some cases lead to death (Orlando *et al.*, 2008; Willis *et al.*, 2008).

Some AAs have metabolic roles proportional to dietary intake. For instance, there is a dose–response relationship between IAA concentration

Table 16.1 Indispensable, dispensable and conditionally dispensable amino acids in the human diet

Indispensable	Dispensable	Conditionally indispensable
Histidine	Alanine	Arginine
Leucine	Aspartic acid	Cysteine
Isoleucine	Asparagine	Glutamine
Lysine	Glutamic acid	Glycine
Methionine	Serine	Proline
Phenylalanine		Tyrosine
Threonine		
Tryptophan		
Valine		

in the blood and muscle protein synthesis, the latter being maximally stimulated with a postexercise consumption of IAAs at a dose of approximately 10 g/day. In particular, leucine has been demonstrated to be a key activator of muscle protein synthesis (Phillips, 2011).

The small intestine is a major site for extensive catabolism of IAAs in humans and animals (Stoll *et al*., 1998), whereas branched-chain AAs (BCAA) are primarily metabolised in skeletal muscle, where they serve both as an important energy substrate and as precursors for the synthesis of other AAs and proteins (Platell *et al*., 2000). BCAAs serve as an oxidative fuel source and stimulate the synthesis of glutamine, an important nutrient for rapidly dividing cells, especially in the gut and immune system. In the brain, BCAAs also act as amino group donors in the synthesis of glutamate, the major excitatory neurotransmitter of the mammalian nervous system and the most important AA neurotransmitter. At least one third of the amino groups of brain glutamate are derived from BCAAs, with leucine donating no less than 25% (Yudkoff, 1997).

The scientific community has reached some consensus on IAAs requirements for men (FAO/WHO/UNU, 2007), whereas in women, infants, children and the elderly, IAAs requirements need to be studied further. This aspect is of key relevance because the indices used for evaluating the nutritional quality of dietary proteins are based on their IAA composition with reference to the recommended requirements. The renewed series of recommended (FAO/WHO/UNU, 2007) requirements for IAAs, together with the AA content of some food sources, are shown in Fig. 16.2.

A detailed description of individual AA requirements for humans at different ages is reported in the Report on Dietary Reference Intakes of the US National Academy of Sciences (2005) and critically discussed in the review by Boutry *et al*. (2008).

16.3 Methods for Evaluating the Nutritional Quality of Dietary Proteins

Assessing protein quality with respect to its efficiency in supporting body protein metabolism should firstly take into consideration the capacity of the protein to provide a suitable source of N and IAAs. For this purpose, the nutritional quality of dietary proteins was classically evaluated with the chemical score (CS) obtained by calculating the content of each IAA of a food source as a percentage of the same AA in a reference protein, such as egg, which is regarded as being well balanced in AA content in relation to human needs. Compared with egg, human milk shows a CS of 100% and cow milk of 95%, with methionine and cysteine being the limiting IAAs. Although this method is fast and cost-effective in comparison to in vivo measurements, its shortcoming is that protein digestibility and AA bioavailability are not taken into consideration. Another limitation is that protein metabolism may be influenced by the excess of other AAs or by the presence of anti-nutritional factors in the food.

A significant improvement in the routine assessment of dietary protein quality took place with the introduction of the PDCAAS (FAO/WHO, 1991). In this case, the CS is calculated for the first limiting AA in the test protein with respect to that of a reference (scoring) pattern. The reference pattern is derived from the IAA requirements of 1–3-year-old children, to cover all ages from 1 year on (Schaafsma, 2000). The value is then corrected for protein digestibility, which is determined in rats over a 5-day period. A value >100%, measured in most animal proteins, means that the protein source

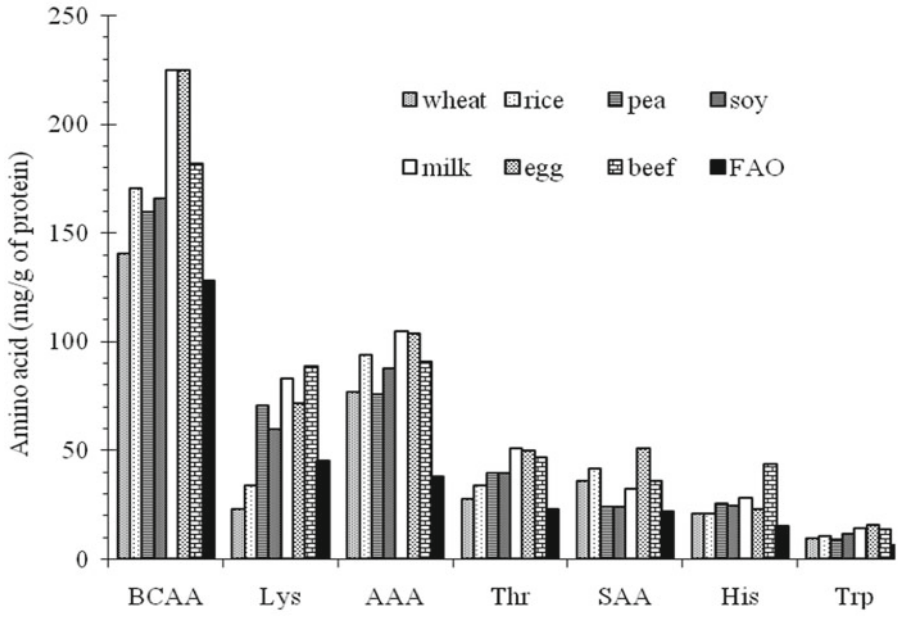

Fig. 16.2 Distribution of indispensible amino acids (IAAs) in food proteins and current dietary IAA requirements recommended by the FAO/WHO/UNU (2007) (*Source*: FAO/WHO/UNU, 2007; Tomé, 2010. *BCAA* branched-chain AAs (leucine, isoleucine, valine); *AAAs* aromatic AAs (phenylalanine, tyrosine); *SAAs* sulphur AAs (cysteine, methionine))

provides the IAAs at a level higher than the requirements. However, calculated scores >100% are truncated so that the capability of a high-quality protein to compensate proteins deficient in one or more IAA is underestimated. For instance, milk has a non-truncated PDCAAS = 120%, casein = 123% and whey protein = 115% (Phillips, 2011), but no discrimination among these products is possible if all values are truncated to 100%. As a consequence, this approach underestimates the power of a high-quality protein to balance the IAA composition of other proteins and thus fails to be useful as a comparative tool.

Despite these limitations, the PDCAAS has been considered the most suitable regulatory method for evaluating the protein quality of foods and infant formulae. Because this method is based on human AA requirements, it is inherently more appropriate than animal assays used for predicting the protein quality of foods (FAO/WHO, 1991). Since 1993, the Food and Drug Administration adopted the PDCAAS as a standard method to calculate protein quality. The validity of the PDCAAS method was endorsed by the FAO/WHO, whereas it has been recommended that the anti-nutritional factors affecting protein digestibility, including those formed during processing, should be investigated further.

16.3.1 Protein Digestibility

A second important issue in nutritional quality evaluation of a protein relates to its bioavailability, or digestibility, or the capacity to provide metabolically available N and AAs to tissues and organs.

Protein digestibility can be evaluated by measuring the free AA (FAA) or specific soluble N fractions released from the test protein after in vitro digestion with one or more proteolytic enzymes under conditions mimicking the in vivo digestive process. Several procedures have been proposed (Mandalari *et al*., 2009), making it difficult to compare results from different authors, whereas a standardised protocol is under study within a project funded by the European Commission (Food and Agriculture COST Action, 2011). Much of the interest in simulated

digestion studies comes from the possibility of assessing the allergenic potential of either intact proteins or the released peptides. This aspect will be addressed in the following sections.

The classical approach for calculating protein digestibility is based on N balance determined in vivo both in animal models (Gaudichon *et al*., 1994) and in humans (Bos *et al*., 1999; Gaudichon *et al*., 1999; Mariotti *et al*., 2001). Basically, the difference between the amounts of ingested and excreted N, expressed relative to ingested N, is obtained through measurements repeated over several days. The excreted N can be calculated from the faecal N ('apparent faecal digestibility', including the N metabolism of the microorganisms present in the large intestine) or from digesta sampled at the end of the small intestine ('ileal digestibility') (Darragh and Hodgkinson, 2000). With respect to milk, Bos *et al*. (1999) recorded similar values of faecal and true ileal digestibility (96.6% and 95.5%, respectively) in human subjects. In any case, ileal digesta also contain a significant proportion of non-dietary AAs from mucus, cells, digestive enzymes and bile (endogenous N), which should be quantified in subjects consuming a protein-free diet. In this regard, a more reliable technique, whose limitation is its high cost, is based on the use of proteins labelled with stable isotopes (^{15}N or ^{13}C). Intrinsically labelled animal or plant proteins can be obtained and adopted for studies on humans (Pennings *et al*., 2011). In this way, it is possible to calculate the 'true faecal digestibility' as $[N_{ingested} - (N_{faeces} - N_{faecal\ endogenous})]/N_{ingested}$.

The true faecal digestibility of protein is considered a good approximation of the bioavailability of AAs of properly processed food products (FAO/WHO, 1991). As shown in Table 16.2, milk and milk products (like other animal proteins) are highly digestible by humans in comparison to plant proteins.

16.3.2 Efficiency of Protein Utilisation

The results of the in vivo tests to evaluate the efficiency of protein utilisation can be expressed through such parameters as the protein efficiency ratio (PER), net protein ratio (NPR), biological value (BV) and net protein utilisation (NPU) (Pellett and Young, 1980). The PER in rats represents the weight gain per gram of protein consumed, evaluated over a period of 28 days. Although time-consuming, the PER has been used extensively for ranking dietary protein sources and for calculating the US Recommended Daily Allowance (USRDA) for labelling regulations. The PER has been criticised for not meeting the criteria of a valid routine test and because modifications in the total food intake give rise to an increase in its levels, thus reducing the capability to compare proteins.

The calculation of the NPR differs from that of the PER in that a protein-free control group is considered. In fact, the NPR is obtained by adding the weight gain, per g of protein consumed, of rats fed the test protein and the weight loss of the animal group fed the protein-free diet. This parameter, measured over a 2-week period, although characterised by a high relationship to protein quality, has the limitation of being a single-dose method (Boutrif, 1991). Both the NPR and the PER values can be corrected to a 0–100 scale, thereby providing the corresponding relative RNPR and RPER indices.

The percentage of $N_{retained}/N_{absorbed}$ gives the BV, which is an expression of the utilisation of the absorbed dietary N, whereas the NPU index allows the evaluation of the ingested amount of N that is retained ($NPU = N_{retained}/N_{ingested}$). True digestibility is reflected in this parameter, being $NPU = BV \times digestibility$. The NPU is assessed from long-term balance measurements in humans.

The whole-body protein balance method, whose classic parameter is the PDCAAS, has been criticised because of the existence of a diurnal cycling of N retention and loss between the fasted and fed states, which leads alternately to postprandial N accretion and post-absorption N loss phases (Bos *et al*., 2000). The acute utilisation of N from a dietary protein may be evaluated by N deposition in the postprandial phase. As the postprandial phase is critical for dietary protein utilisation, the measurement of the immediate retention of dietary N following meal

Table 16.2 True faecal digestibility and PDCAAS values of milk, milk products and other dietary sources

Protein source	Digestibility (%)	PDCAAS (%)
Milk and milk products		
Milk	94[a]–95[b,c]	100[b]
Casein	95[b,c]–99[a]	100[b]
Whey protein	98[b]	100[b]
Whey protein concentrate	100[d]	100[d]
Whey protein hydrolysate	99[d]	100[d]
Skim milk powder	93[d]	100[d]
Others		
Egg	97[b,c]	100[b]
Beef salami	99[e]	100[f]
Tuna	94[e]	100[f]
Soy	91[b]	99[b]
Wheat	86[b]	54[b]
Rice	88[b]	55[b]

Sources: [a]Sarwar (1997), [b]AFSSA (2007), [c]Sarwar Gilani *et al.* (2005), [d]Gilani and Sepehr (2003), [e]Sarwar *et al.* (1989), and [f]Schaafsma (2005)

ingestion (net postprandial protein utilisation, NPPU) represents a reliable approach for evaluating dietary protein efficiency (Tomé, 2010). Assessment of the NPPU of dietary proteins may represent an appropriate approach for the further validation of the PDCAAS scoring method because the latter parameter is known to be influenced by protein turnover. The non-truncated PDCAAS values reported by Tomé and Bos (2000) for milk, soy, pea and wheat (120, 99, 73 and 36, respectively) and the NPPU values reported by Fouillet *et al.* (2002) for the same proteins (75, 71, 71 and 62, respectively) are in quite good agreement, confirming the capability of the NPPU method to discriminate protein quality (Reeds *et al.*, 2000). The measurement of short-term retention of dietary N (Bos *et al.*, 1999) is made possible by the use of ^{15}N-labelled proteins.

Tomé (2010) reported that, in humans adapted to a normal protein diet (1 g/kg/day), about 65–80% of dietary N is retained 12 h after a mixed meal. In particular, 30–50% is distributed in the splanchnic tissues (i.e. intestine, liver, etc.) and 20–30% in peripheral tissues (muscle, skin, etc.). The wide range of reported values is due to the different kinetics of dietary N distribution in the anabolic and catabolic pathways of different tissues after protein ingestion.

16.4 Nutritional Quality of Proteins in Milk and Milk Products

Cow milk is considered a source of dietary protein with excellent nutritional value (Tomé, 2010). Such quality, ascribed to the supplementation of both N and IAAs necessary to meet human requirements, is also attributable to the presence in milk of a wide range of biologically active proteins and peptides having specific nutritional and physiological properties (Bosze, 2008). However, technological processes used in the manufacture of milk products may impair this value. All of these topics are addressed in the following sections.

16.4.1 Amino Acid Composition

The AA composition of milk proteins is reported in Table 16.3. The IAA composition of milk proteins is similar to that of other animal proteins, such as egg, but with limited amounts of sulphur-containing AAs (SAA). Despite this limitation, the IAA pattern of milk abundantly covers the requirements defined by the FAO/WHO/UNU to satisfy AA needs in adults. The IAA content of milk proteins, expressed as a percentage of the daily IAA requirement, when protein is supplied

Table 16.3 Amino acid composition (% of total AAs) of milk proteins

Amino acid	Casein	Whey protein
Aspartic acid/asparagine	7.1	10.5
Threonine	4.9	7.0
Serine	6.3	4.8
Glutamic acid/glutamine	22.4	17.6
Proline	11.3	5.9
Glycine	2.7	1.8
Alanine	3.0	4.9
Cysteine	0.34	2.3
Methionine	2.8	1.7
Valine	7.2	5.7
Isoleucine	6.1	6.4
Leucine	9.2	10.3
Tyrosine	6.3	2.9
Phenylalanine	5.0	3.1
Tryptophan	1.7	2.4
Lysine	8.2	8.7
Histidine	3.1	1.7
Arginine	4.1	2.3

at the mean requirement (i.e. 0.66 g/kg/day), demonstrates that milk represents a well-balanced food source. In particular, the Expert Consultation of the FAO/WHO/UNU (2007) calculated that lysine, SAAs and aromatic AAs (AAA) represent more than 150% of the daily requirements and tryptophan is the most relatively abundant IAA (417% of the required value). Furthermore, milk can fully satisfy all IAA requirements when ingested as the only protein source (Reeds *et al*., 2000), and in a mixed diet, milk can be considered complementary to dietary sources lacking threonine and especially lysine, such as grains.

Milk proteins consist mainly of casein (~80%) and whey protein (~20%), having different AA compositions (Table 16.3). In particular, whey protein has higher amounts of SAAs (methionine and cysteine), lysine, threonine and tryptophan (Fig. 16.3). Moreover, whey proteins have a slightly higher true ileal digestibility for all AA. In fact, whey protein is a mixture of α-lactalbumin (α-la), β-lactoglobulin (β-lg), immunoglobulins (Ig), blood serum albumin (BSA) and perhaps other components, including glycomacropeptide (GMP) (which is cleaved from κ-casein by chymosin during cheese production), each of which has a distinct AA composition. Knowing the nutritional and physiological roles of the different AAs, it would be possible to develop functional foods based on milk proteins having AA compositions suitable to meet the needs of people interested in controlling diabetes, losing fat or building muscle protein (Etzel, 2004). α-La, for example, has a high cysteine content, and β-lg is rich in glutamine, whereas GMP has an extraordinarily high threonine content and completely lacks cysteine, histidine, arginine, tyrosine, tryptophan and phenylalanine. It has been demonstrated that GMP, when supplemented with IAAs, may represent a safe and highly acceptable primary protein source in the nutritional management of phenylketonuria (Ney *et al*., 2009).

Among the different protein sources, milk is relatively abundant (21%) in BCAA, and leucine represents 10% of the total AAs and approximately 50% of the BCAAs (Table 16.4).

As already mentioned, in addition to protein synthesis, BCAAs play important roles in many metabolic functions. Specifically, leucine is a key activator in regulating the turnover of muscle proteins (Rennie *et al*., 2006). Because of their high content of leucine, compared with other proteins (Table 16.4), milk and whey proteins are

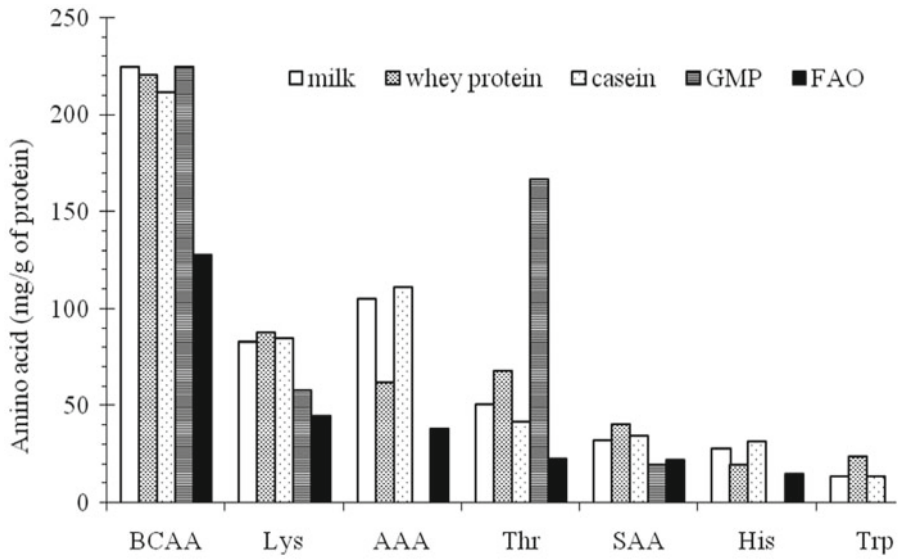

Fig. 16.3 Distribution of indispensible amino acids (IAAs) in milk, milk protein fractions and glycomacropeptide (GMP) in comparison to human requirements recommended by the FAO/WHO/UNU (2007) (*Source*: FAO/WHO/UNU, 2007; Boutrif, 1991; USDEC, 2011; Etzel, 2004)

Table 16.4 Content of leucine and branched-chain amino acids (BCAA) (leucine, isoleucine and valine) in milk and other food sources

	Leucine	BCAA
Protein	(g/100 g protein)	
Whey isolate	14	26
Milk	10	21
Egg	8.5	20
Soy isolate	8	18
Navy beans	7.6	16
Whole wheat flour	7	15

Sources: Layman and Baum (2004) (source: USDA Food composition tables) and Young and Pellet (1990)

receiving much attention for the nutrition of athletes (Phillips, 2011) and also in clinical nutrition of the elderly to prevent sarcopaenia (Paddon-Jones and Rasmussen, 2009). The main unanswered questions in this regard are how the source of dietary protein and the degree of hydrolysis of the ingested protein can affect increases in muscle protein synthesis. Consumption of hydrolysed whey protein results in a rapid and pronounced rise in aminoacidaemia and leucinaemia, compared with isonitrogenous quantities of both casein and soy (Phillips, 2011). These findings are apparently in contrast with those obtained by whole-body measurements (Lacroix *et al*., 2006), but it is useful to recognise that only 25% of the whole-body response is due to muscle protein. Currently, hydrolysed whey protein and/or casein is largely used in the formulation of sports/recovery integrators.

The SAAs, methionine and cysteine, which represent approximately 3% of milk proteins, are important for the synthesis of proteins in the immune system (Grimble, 2006).

Methionine is a methyl group donor that participates in the methylation of DNA and proteins, the synthesis of spermidine and spermine and the regulation of gene expression (Wu *et al*., 2006). Furthermore, methionine is a substrate for the synthesis of choline and, thus, phosphatidylcholine and acetylcholine, which are essential for nerve function and leucocyte metabolism (Kim *et al*., 2007a). Cysteine is the precursor of glutathione (GSH), which scavenges free radicals (e.g. hydroxyl radical) and other reactive oxygen species (e.g. H_2O_2), and conjugates with various electrophiles and xenobiotics for their detoxification (Fang *et al*., 2002). According to

Fratelli *et al*. (2005), the intracellular concentrations of GSH play an important role in regulating cellular signalling pathways in response to immunological challenges. Furthermore, GSH concentrations in antigen-processing cells modulate immune responses, including antibody production (Peterson *et al*., 1998). Nowadays, a number of whey products rich in BCAAs and SAAs are commercially available, including whey protein concentrates, whey protein isolate, reduced lactose whey, demineralised whey and hydrolysed whey, and many clinical trials have demonstrated the efficacy of these products in the treatment of several diseases, such as cancer, AIDS, hepatitis B, cardiovascular disease and osteoporosis (Marshall, 2000). Whey protein also acts as an antimicrobial agent and enhances athletic performance.

Bovine milk contains a small amount of FAAs, which represent a ready source of substrate for bacterial growth. Table 16.5 reports the FAA content in raw bovine milk compared to that of human milk as reported by Agostoni *et al*. (2000). Human milk contains five times the amount of FAAs in bovine milk and is thus more easily metabolised by the newborn.

16.4.2 Nutritional Quality, Digestibility and Utilisation

The nutritional quality of milk proteins, as well as that of its two main fractions (i.e. casein and whey protein), has been evaluated by several authors using different indices. The measured values are compared with those obtained for other dietary proteins in Table 16.6. With respect to grains and legumes, milk proteins are characterised by higher BV values, suggesting a better utilisation of the absorbed dietary N, and higher NPU values, which reflect true digestibility. Furthermore, all of these indices confirm the higher nutritive value of the whey protein fraction with respect to casein.

Milk proteins are characterised by a high NPPU in humans, that is, the amount of dietary N retained in the body after milk protein ingestion, showing that milk proteins are characterised by a well-balanced AA composition with respect to human needs. The data in Table 16.6 suggest that the type of protein can specifically influence the kinetics and the profile of AA delivery, which influences protein metabolism and N retention.

The quality of selected milk protein fractions has been assessed using indices based on rat growth (Table 16.7).

The RNPR values were higher than those of RPER because the former index considers protein used for both growth and maintenance. The PDCAAS values of the different milk protein fractions are identical, all of them being truncated to 100. Casein integrated with methionine increases the scores for all of the indices.

Casein and whey protein fractions are digested differently. Casein shows a delayed gastric emptying due to its clotting in the stomach. In contrast, whey protein remains soluble and is evacuated more rapidly so that AA delivery to the gut is more rapid. As a result, casein provokes a smaller postprandial increase in plasma AA compared with the non-coagulating whey protein. To describe the digestion and absorption of casein and whey proteins, Boirie *et al*. (1997) introduced the concept of 'slow' (slowly digested and absorbed) and 'fast' (rapidly digested and absorbed) proteins. These authors reported that slow proteins, such as casein, sustain a better N utilisation than fast proteins and are less satiating. Deglaire *et al*. (2008) recorded a similar nutritional value for intact casein (a slow protein) and hydrolysed casein (a fast protein), in agreement with the N balance studies of other authors (Sales *et al*., 1995) but apparently in contradiction with the results of Boirie *et al*. (1997). Indeed, the data reported by Deglaire *et al*. (2008) suggest that the correlation between digestion/absorption rates and net protein retention is more complex than that based on the slow/fast protein concept. Lacroix *et al*. (2006) investigated the postprandial kinetics of dietary N after the ingestion of different milk protein fractions in humans. The authors demonstrated that the stimulation of peripheral anabolism under the examined conditions is unlikely to occur after ingestion of whey protein isolate, despite its high leucine content (Table 16.4). A synergistic effect between casein and whey protein in terms of metabolic utilisation was observed. Indeed, Lacroix *et al*.

Table 16.5 Average free amino acid content (mg/L) in bovine and in human milk

Amino acid	Bovine milk[a] ($n=12$)	Human milk[b] ($n=40$)
Aspartic acid	3.3	24.4
Threonine	1.4	11.6
Serine	1.0	28.8
Asparagine	0.1	n.d.
Glutamic acid	40.0	174.2
Glutamine	1.4	41.6
Glycine	6.3	9.4
Alanine	3.5	20.3
Valine	3.2	8.5
Methionine	0.1	1.3
Isoleucine	0.5	4.4
Leucine	0.8	7.3
Tyrosine	1.0	0.5
Phenylalanine	0.5	3.9
Lysine	3.0	5.7
Histidine	0.4	1.2
Arginine	2.7	6.2
Proline	2.1	7.4
Total free amino acids	71.3	356.7

Sources: [a]Personal data and [b]adapted from Agostoni *et al.* (2000)

Table 16.6 In vivo indices of protein quality in milk and other food sources

Protein source	PER	BV %	NPU %	NPPU % humans
Milk	2.5[a]	91[a]–93[b]	77[c]–82[c,a]	75[d]–81[d,c]
Casein	2.5[a]	75[b]–77[a]	76[a]–79[c]	71[d]
Whey protein	3.2[a]	100[b]	92[a]–95[c]	64[d]
Others				
Egg	3.9[a]	100[b]	94[a]	
Beef	2.9[a]	75[b]–80[a]	73[a]	73[d]
Soy	2.2[a]	74[a]	61[a]	72[d]
Wheat	0.8[a]	64[a]	67[a]	66[d]

Sources: [a]Hoffman and Falvo (2004) and FAO-WHO (1973), [b]National Research Council (1989), [c]Bos *et al.* (1999), and [d]AFSSA (2007)
PER protein efficiency ratio; *BV* biological valve; *NPU* net protein utilisation; *NPPU* net postprandial protein utilisation

Table 16.7 Values of protein quality indices of selected milk protein fractions

Protein source	PER	NPR	RPER (%)	RNPR (%)
Skim milk	3.7	4.7	77	82
Casein	3.9	4.8	80	84
Casein + met	4.8	5.7	100	100
Lactalbumin	4.3	5.2	89	91

Sources: Sarwar (1997) and Gilani and Sepehr (2003)
PER protein efficiency ratio; *NPR* net protein ratio; *RPER* relative protein efficiency ratio; *RNPR* relative net protein ratio

(2006) measured that the dietary N utilisation in a milk-based meal was better, or at least identical, to that in the casein and whey protein meals. The higher value of the milk-based meal was suggested by the authors to be due to the combination of an early metabolic and hormonal stimulation by the whey protein fraction and a sustained delivery of AAs from casein. A better NPPU in terms of muscle protein accretion seems to be induced by whey proteins compared to either casein or casein hydrolysate in healthy older men (Pennings *et al*., 2011) and athletes (Phillips, 2011). Such results have led to whey proteins becoming an important nutritional and functional food ingredient, with extensive use in such food applications as sport beverages, meat replacement products and energy bars.

Fouillet *et al*. (2002) developed a compartmental model mimicking dietary N absorption, elimination and distribution throughout the body after the ingestion of a meal containing either milk or soy. A lower whole-body N retention of soy proteins compared to milk proteins was observed. The NPPU values 8 h after the meal were 80% and 72% for milk and soy, respectively. In addition, the kinetics of dietary N absorption and subsequent transfer to peripheral and splanchnic tissues was differentially affected by the protein source. Milk caused lower ileal losses and splanchnic oxidation in comparison to soy. According to the predictions of the model, 8 h after a meal, dietary N incorporated into proteins differed significantly for milk and soy meals, being 26% and 19% of the ingested N, respectively. Actually, Fouillet *et al*. (2002) ascribed the higher efficiency of peripheral protein synthesis after milk ingestion to the higher proportion of BCAAs. Similarly, Kimball and Jefferson (2001) hypothesised a higher amount of BCAAs, which are known to be stimulators of muscle protein anabolism, in the peripheral area after milk ingestion. Bos *et al*. (2003) concluded that the differences in the NPPU of milk and soy proteins are mainly due to differences in digestion kinetics but that AA composition may also play a role.

Milk protein hydrolysates are largely used in the formulation of clinical diets in the case of food allergies caused by intact protein epitopes. In vivo studies demonstrated that hydrolysis does not affect the nutritional quality of milk proteins. In vitro studies (Syndayikengera and Xia, 2006) on sodium caseinate and whey protein concentrate showed an improved enzymatic digestibility (by pepsin/pancreatin) of both hydrolysed forms. Specifically, in terms of AA composition, CS and BV, whey protein concentrate and its hydrolysate have higher nutritional values than do sodium caseinate and its hydrolysate. Boza *et al*. (1994), comparing the nutritional values of whey protein and casein with their respective hydrolysates, concluded that enzymatic hydrolysis per se does not significantly decrease the nutritional value of milk proteins. Actually, hydrolysis seemed to improve the NPU and BV of whey proteins.

16.4.3 Effects of Processing

Nowadays, milk proteins are introduced in the human diet almost exclusively via processed milk products. Thus, evaluation of the nutritional quality of milk proteins must consider the effects of processing treatments on the bioavailability of both N and AA.

This section deals with the impact of the most common technological treatments employed in milk processing (Table 16.8) on the nutritional quality of milk proteins in the finished products.

16.4.3.1 Heat Treatments

Essentially all milk products are submitted to heat treatments during processing, with the aim of ensuring safety and stability, as well as for many other technological reasons. Moreover, in addition to heating during processing, storage may be regarded as an additional heat treatment, especially for those milk products (e.g. sterilised or powdered milk, infant formulae) usually stored at room temperature for an extended period of time.

The heating of milk promotes several reactions that induce protein modifications, which may affect the nutritional quality of milk. From a nutritional point of view, the most important changes induced by heat are related to (1) protein denaturation, (2) protein glycation, (3) β-elimination reactions, (4) AA racemisation and (5) formation of isopeptide bonds (Pellegrino *et al*., 1995).

Table 16.8 Main processes applied in dairy industry

Milk product	Process
Drinking milk	Pasteurisation
	UHT
	In-bottle sterilisation
	Homogenisation
Cheese	Pasteurisation of cheese milk
	Coagulation
	Fermentation
	Ripening
Processed cheese	Melting
	Sterilisation
Fermented milk	Heat treatment of milk
	Fermentation
Powdered milk products	Heat treatment
	Concentration by evaporation
	Drying

1. Among milk proteins, thermal denaturation affects mainly whey proteins. No structural modifications of casein occur upon heating at the conditions usually applied in industrial processing as the protein lacks secondary and tertiary structures. Upon heating, the native globular structure of whey proteins undergoes unfolding, exposure of thiols and consequent formation of both whey protein-whey protein and whey protein-casein aggregates. The effect of heat-induced denaturation on the digestibility of milk proteins has been evaluated in milk products submitted to different heat treatments (pasteurisation, UHT sterilisation, in-bottle sterilisation) by many researches using different criteria and conditions. This may partly explain why contradictory results are reported in the literature. Some authors (Rudloff and Lonnerdal, 1992; Carbonaro *et al*., 1996; Alkanhal *et al*., 2001) reported that the formation of protein aggregates *via* direct covalent interactions and the consequent loss of solubility seem to decrease protein digestibility, mainly because digestive enzymes may no longer recognise their specific substrate or cannot reach their site of action. Lacroix *et al*. (2008) evaluated the effect of pasteurisation and UHT treatment on the protein digestibility of skimmed milk in humans. This study revealed that pasteurised milk had a higher NPPU value than that measured in UHT milk (76.2% vs. 68.3%). On the other hand, Singh and Creamer (1993) highlighted an easier proteolysis of whey proteins when they lose the native globular structure. It has been reported (Hiller and Lorenzen, 2010) that glycation by complex sugars (i.e. dextran) may facilitate the unfolding of β-lg, resulting in easier access to peptide bonds by proteolytic enzymes. Lacroix *et al*. (2008) observed that the digestive kinetics are more rapid for UHT milk than for pasteurised milk, leading to an increased transfer of dietary N into plasma protein or urea. Indeed, interaction between whey protein and casein upon heat treatment results in a softer casein coagulum and in a higher susceptibility to proteolytic enzymes, with a consequent acceleration of protein digestion. Moreover, a large increase in very small easily digestible casein micelles in UHT milk could explain the improvement of protein digestibility. The improving effect of heat treatment on milk protein digestibility was also pointed out by Kim *et al*. (2007b).
2. Upon heating, milk protein lactosylation induced by the Maillard reaction (MR) may occur. The MR is considered the most important mechanism impairing the quality of milk proteins during the different technological treatments. The main nutritional consequence of the MR in milk is the loss of bioavailable IAAs, among which lysine is the most affected, due to both its abundance in milk proteins and the high reactivity of its ε-amino group towards lactose. During heat treatment of milk, lysine is blocked in the Amadori compound (lactulosyl-lysine in milk) deriving from the condensation reaction between the ε-amino group of lysine and the glucose moiety of lactose. Characteristic levels of blocked lysine in heated milk and in dairy products are reported in Table 16.9. These data show that the loss of bioavailable lysine can be considered negligible in pasteurised or UHT milk, whereas higher levels with some nutritional significance can be encountered in in-bottle sterilised milk, in processed cheese and in powdered milk products. In particular, Cattaneo

Table 16.9 Levels of blocked lysine in some dairy products (Pellegrino *et al.*, unpublished)

Type of product/process	Blocked lysine (% total lysine)
Drinking milk	
pasteurised	0.1–0.2
UHT	3.0–6.5
in-bottle sterilised	11.0–13.5
Cheese	
Fresh	0.2–0.3
Processed	6.5–8.7
Skim milk powder	7.2–9.8
Milk based infant formulas	
liquid	11.3–12.5
powder	18.5–31.2
Yogurt	3.5–4.9

et al. (2009) demonstrated that by adopting a conventional UHT treatment and optimising the processing conditions for liquid infant formulae, the loss of available lysine can be dramatically reduced. It is noteworthy that FAAs can also be potentially involved in the MR. Thus, the extent of blockage of IAAs should be evaluated taking into account the levels of FAAs. In this regard, Rerat *et al*. (2002) showed that the decreased bioavailability of IAA other than lysine plays a minor role in the loss of the nutritional value of milk, infant formulae and other foods subjected to heat treatments.

Besides the loss of IAAs, the nutritional quality of milk proteins may be reduced by newly formed molecules arising from the advanced MR. Some of these compounds have been demonstrated to inhibit in vitro digestion by carboxypeptidase A and aminopeptidase M (Oste *et al*., 1987), thus affecting the utilisation of dietary protein. In this regard, Gilani and Sepehr (2003) suggested that the presence of Maillard products reduced the protein digestibility of skim milk powder.

In addition, protein digestibility may be reduced by polymerisation induced by the MR. Although the polymerisation phenomena of milk proteins have been extensively studied and related to the formation of dicarbonyl compounds, until now the only identified cross-linker arising from the MR in food was pentosidine. However, only traces of this compound were found in sterilised milk products, whereas higher amounts were detected in roasted coffee and bakery products (Henle *et al*., 1997). A recent study (Hiller and Lorenzen, 2010) reported the formation of highly cross-linked protein/sugar complexes that sterically shield peptide bonds from proteolysis. This phenomenon could explain the impaired in vitro digestibility of sodium caseinate submitted to heat treatment.

3. Along with cross-linking phenomena deriving from the MR, several cross-linkers are reported to form *via* β-elimination reactions mainly involving phosphoserine and cysteine with the formation of the key intermediate compound dehydroalanine. Further condensation reactions lead to formation of AAs, which do not occur naturally, such as lysinoalanine (LAL), lanthionine, ornithinoalanine and histidinoalanine. Among these, LAL has been studied extensively (Friedman, 1999a) in food proteins because of its suspected role in inducing nephrocytomegaly in rats. However, up to now, no evidence of this effect has been observed in primates. The presence of LAL has been demonstrated in processed cheese (Pellegrino *et al*., 1996), in drinking milk (Faist *et al*., 2000; Cattaneo *et al*., 2008a) and in liquid infant formulae (Cattaneo *et al*., 2009).

The effect of cross-linking arising from β-elimination reactions on the nutritional quality of protein content can be considered similar to that of cross-linkers formed via the MR. In this regard, Gilani and Sepehr (2003) reported that the digestibility of α-la sensitively decreased when it was submitted to alkalinisation followed by a heat treatment promoting the formation of significant amounts of LAL. However, some authors (de Vrese *et al*., 2000) reported that the decrease in casein and β-lg digestibility subsequent to alkalinisation and heat treatment was mainly due to AA racemisation to the D form and only partially due to LAL formation. Moreover, it was demonstrated (Pellegrino *et al*., 1999) that compounds other than LAL and pentosidine are responsible for heat-induced β-casein covalent cross-linking.

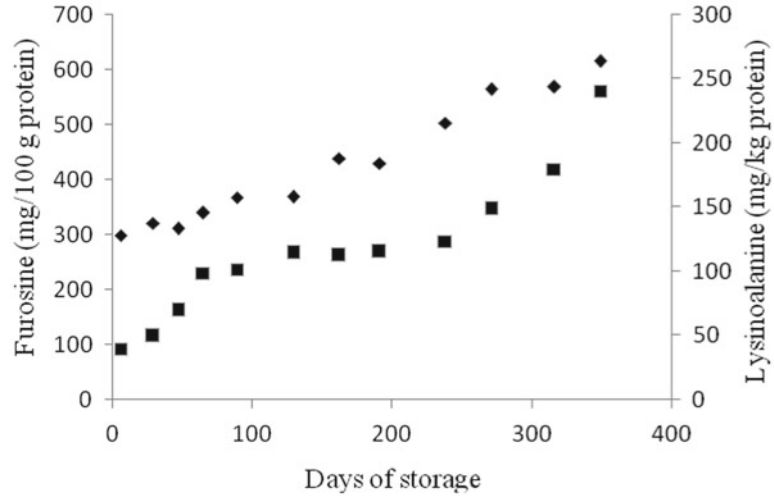

Fig. 16.4 Accumulation of furosine (◆) and lysinoalanine (■) in liquid milk-based infant formulae during storage at 20°C (adapted from Cattaneo *et al*., 2009)

For a comprehensive evaluation of the nutritional significance of the presence of LAL in food, it is useful to consider that the formation of LAL results in a loss of such IAAs as lysine, cysteine and threonine, although, as far as conventional dairy products are concerned, the levels of lysine involved in LAL formation are significantly lower than those blocked in the early formed MR compounds.

Prolonged storage of several dairy products at ambient temperature also affects the nutritional quality of their protein content. During storage, further protein modifications occur as demonstrated by the progressive accumulation of some molecular markers of heat load, such as furosine (arising from early MR) and LAL in milk-based infant formulae reported in Fig. 16.4. These products are usually stored at room temperature for 8–12 months if liquid or up to 24 months if powdered, and thus, their nutritional quality may be greatly impaired by adverse storage conditions (Pellegrino *et al*., 2011). The same behaviour of the above-mentioned heat-load molecular markers was observed by Cattaneo *et al*. (2008a) during storage of conventional UHT milk. Results arising from this research may support the findings of Alkanhal *et al*. (2001), who observed a decrease in the nutritional quality of proteins in UHT milk after a 3-month storage at ambient temperature.

4. During heat treatment of protein, especially at alkaline pH, AA residues may undergo racemisation from L- to D-enantiomers. The presence of D-AAs lowers the nutritional quality of proteins; in fact, the hydrolysis rates of D-AA-containing proteins are slower than those of unmodified proteins because peptide bonds involving D-AAs are not easily cleaved by proteolytic enzymes. Moreover, racemisation can lead to formation of non-metabolisable and biologically non-utilisable forms of AAs, and nutritionally antagonistic and toxic compounds (Friedman, 1999b). Unlike most mammalians, humans lack specific biochemical pathways for converting some D-AAs into the corresponding L-enantiomers (Meade *et al*., 2005). Raw milk contains some D-AAs (D-alanine, D-aspartic acid, D-glutamic acid, D-lysine and D-serine), derived from lactic acid bacterial cell walls and from cow rumen microorganisms (Marchelli *et al*., 2008). The content of these AAs is not considered important from a nutritional point of view, and even milk pasteurisation and UHT treatment does not

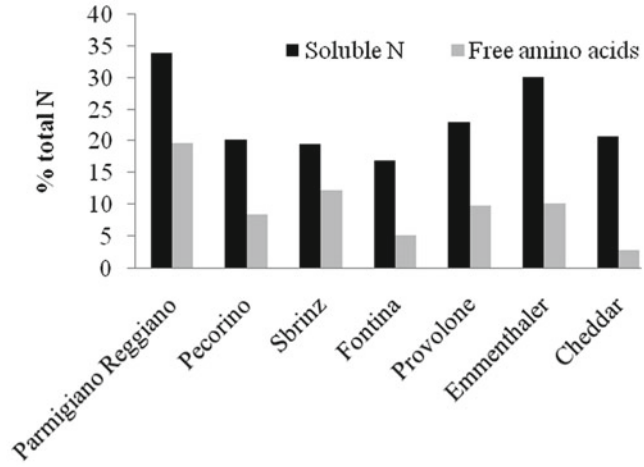

Fig. 16.5 Levels of soluble N and free amino acids in selected cheeses (Pellegrino *et al.*, unpublished)

result in a significant increase in the level of D-AAs (Csapó *et al.*, 2007).

5. Severe heat treatments of milk may also lead to the formation of peptide bonds between the ε-amino group of lysine and the amino group of asparagine or glutamine. The isopeptides involving glutamine and lysine are fully bioavailable, and their formation prevents lysine from reacting *via* the aforementioned reactions that may impair the nutritional quality of milk proteins (Friedman, 1999a).

16.4.3.2 Cheesemaking

Following acid or rennet coagulation, about 95% of casein is transferred from milk to cheese and about 95% of whey protein passes from milk to whey. Because whey proteins have a higher BV than casein, mainly due to the presence of SAAs, cheesemaking implies a slight decrease in the nutritional value of milk proteins in terms of AA composition. From this point of view, the nutritional value of cheese protein can be improved by applying processing technologies capable of increasing whey protein retention in the curd (e.g. ultrafiltration of cheese milk).

An overall evaluation of the nutritional quality of milk proteins in cheese should also consider their increased digestibility. During cheese ripening, a progressive degradation of casein occurs as a result of the alternate activity of proteolytic enzymes deriving from milk, rennet and starter and non-starter bacteria. Indeed, in ripened cheeses, the water-insoluble casein is partly already converted into water-soluble N compounds, such as small peptides and FAAs, which are readily absorbed through the small intestine. For this reason, proteolysis occurring during cheese ripening can be regarded as a sort of predigestion and some varieties of cheese show a higher protein digestibility than whole milk. In Fig. 16.5, levels of FAAs and soluble N fractions in selected cheeses are reported.

Free D-AAs are often reported to be present in long-ripened cheeses, and because, as previously reported, the presence of D-AAs depresses the nutritional quality of proteins, they should not be considered in the total amount of FAAs. Gouda, Emmental, Grana Padano and Parmigiano-Reggiano are reported to contain relatively high amounts of D-valine, D-isoleucine, D-serine, D-alanine, D-glutamic acid and D-asparagine (Friedman, 1999b; Marchelli *et al.*, 2008). The level of D-AAs in ripened cheese seems to be related to the presence of racemases derived from lysis of bacterial cell walls in both the retardation and the death phases (Csapó *et al.*, 2007).

It is noteworthy that, during cheese ripening, after their liberation from casein, several FAAs may undergo degradation reactions that lead to the formation of nonprotein AA (Krause *et al.*, 1997; Cattaneo *et al.*, 2008b), which play important roles in diverse physiological processes. Arginine may be degraded into ornithine and then transformed into citrulline, an immediate precursor for arginine synthesis in virtually all cell types (Wu and Morris, 1998). Decarboxylation of glutamate produces γ-aminobutyric acid (GABA),

an inhibitory neurotransmitter in the central nervous system that has been shown to reduce blood pressure (Nomura *et al*., 1998). Like arginine degradation, glutamate decarboxylation has been interpreted as a response of lactic acid bacteria to high acidity (Cotter and Hill, 2003). The content of GABA determined by Nomura *et al*. (1998) in several commercial cheese types ranged from 4 mg/kg cheese for Edam to 177 mg/kg cheese in Gouda, representing 0.1% and 6%, respectively, of the total FAA content. In Grana Padano and Parmigiano-Reggiano cheeses, GABA does not exceed 0.5% of the total FAA content, roughly corresponding to 400 mg/kg cheese, and higher levels indicate the presence of such defects as late blowing (Cattaneo *et al*., 2008b). Furthermore, in the natural whey cultures commonly used as starters in the processing of hard cheeses, free GABA contents of up to 80 mg/L were observed, representing up to 17% of the total FAA content (personal data, 2011). It remains questionable, however, if supplementation of GABA from milk or dairy products may affect GABA levels in the brain because this AA is not transported efficiently across the blood–brain barrier. Therefore, most of the GABA found in the brain is produced there (Petroff, 2002).

Decarboxylation of FAAs occurring in some long-ripened or moulded cheeses may lead to the formation of amines, among which biogenic amines have been demonstrated to be biologically active, including either hypertensive (tyramine and phenylethylamine) or hypotensive (histamine) effects on blood pressure (Krause *et al*., 1997). Healthy persons, however, rapidly metabolise ingested biogenic amines by converting them into aldehydes and carboxylic acids (Renner, 1993).

16.4.3.3 Other Milk Treatments

The digestibility of the proteins in fermented milk products is enhanced by (1) the partial proteolysis performed by proteases from starter bacteria leading to the release of peptides and AA and (2) the presence of acid-coagulated protein that implies the formation of a softer clot in the stomach than that deriving from liquid milk ingestion. This coagulum is more easily accessed by gastric proteases during digestion (Hewitt and Bancroft, 1985). However, Gaudichon *et al*. (1995), measuring endogenous N secretion after ingestion of yogurt and milk, concluded that fermentation tends to delay the gastric emptying rate.

Homogenisation is usually applied in the processing of drinking milk to assure stability of fat emulsion during storage. Despite the fact that a reduction in fat globule size seems to improve milk fat digestibility, the high pressures (10–25 MPa) applied in milk homogenisation induce protein aggregation (Meade *et al*., 2005) and adsorption of casein micelles and whey protein to the milk fat globule surface, and the impact of this phenomenon on protein digestibility is far from clear (Michalski and Januel, 2006).

16.5 Nutritional and Physiological Activities of Milk Proteins

The nutritional role of milk proteins has been generalised by studying the whole protein supply provided by milk. In the last several decades, simple and cost-effective industrial-scale methods have been developed for separation, fractionation and isolation of the individual proteins occurring in bovine milk and colostrum. These methods have been addressed to maintain some of their biological effects and digestibility. As a consequence, milk proteins are nowadays available for specific applications in different multifunctionality forms, characterised by specific technological properties (e.g. water solubility) and nutritional or sensorial characteristics. These facts have shifted research towards the study and exploitation of the physiological health benefits of individual milk proteins, some of which are of no significance in the principal milk products. Despite many nutritional issues remaining unanswered, milk proteins provide a wide range of important functional and biological properties that are continuously being discovered and for which scientific data are being accumulated. For this reason, some milk proteins have already found applications in the development and manufacturing of novel foods designed to provide health-promoting functions, including prevention of diseases and improvement of consumers' wellbeing. In this regard, the potential biological activities relating to absorption of nutrients,

mineral binding, immune system modulation, antimicrobial and antiviral actions and anticarcinogenic features have been extensively studied.

The nutritional role of caseins has been referenced in terms of their capacity to carry calcium and phosphate. Whey proteins were also suspected to play a role in mineral absorption (Pantako *et al*., 1992; Barth and Behnke, 1997) because several of them can bind minerals and vitamins. For instance, β-lg contains a hydrophobic pocket that, in vitro, can bind to and act as a carrier of different substrates, including retinol, vitamin A and vitamin D, thus simplifying their absorption (Wang *et al*., 1997; Beaulieu *et al*., 2006). The ability of β-1g and α-la to bind cations has been reported as well (Baumy and Brule, 1988; Jeyarajah and Allen, 1994; Simons *et al*., 2002). Lactoferrin is believed to play a role as an iron-binding protein that could be involved in iron transport (Bos *et al*., 2000; Iyer and Lonnerdal, 1993). Other minor whey proteins are responsible for binding and transporting folate, vitamin D, cobalamin and riboflavin (Fox and Kelly, 2003).

As far as physiological activities are concerned, milk proteins are suspected to modulate differentially the release of gastrointestinal hormones that control both gastric and intestinal motilities, as well as gastric and biliopancreatic secretions (Hara *et al*., 1992). Moreover, whey proteins and caseins can affect the synthesis of metabolic components (glutathione, serotonin) or different factors involved in the regulation of the cellular signalling in different tissues and organs (McIntosh *et al*., 1998). As a consequence of these activities, milk proteins influence food and energy intake, glucose metabolism, fatty acid and adipose tissue metabolism or insulin secretion and sensitivity. However, these effects are likely due to the presence of specific bioactive peptides released during digestion of milk proteins and acting either at the luminal level or after absorption (Daniel *et al*., 1990; EFSA, 2009). In this regard, the rate at which milk proteins are digested in the stomach could explain why casein stimulates endogenous N secretions more efficiently than whey protein, as observed by different authors (Mahé *et al*., 1995, 1996). As previously mentioned, the rate at which peptides and AA are released during digestion may differ among the milk proteins (Boirie *et al*., 1997). Casein forms a clot that slowly empties from the stomach, whereas whey proteins are rapidly digested resulting in high concentrations of AAs in the blood stream (Lacroix *et al*., 2006). Nonetheless, different whey proteins are quite resistant to digestion and are still detected in the intestinal lumen following milk ingestion (Mahé *et al*., 1991; Roos *et al*., 1995). Moreover, transcytosis of the intact form of some of these proteins has been demonstrated, as well as their presence in the blood stream after ingestion (Caillard and Tomé, 1994).

There is now a substantial body of evidence suggesting that the major components of bovine milk, as well as several highly purified individual constituents or subfractions, can regulate immune function in nonruminant as well as ruminant species (Cross and Gill, 2000). Whey proteins have been reported as capable of modulating nonspecific and specific immune responses, in both in vivo and in vitro experiments (Gomez *et al*., 2002). On the contrary, Brix *et al*. (2003) demonstrated that β-lg per se did not possess immunomodulatory activity. Eventually, the effect was found to be caused by the endotoxin contamination present in some commercial β-lg preparations. Whey proteins are SAA-rich proteins and have been reported to possess anticarcinogenic properties. Indeed, through provision of methionine and cysteine, they have a positive influence on cellular methylation, and, by stabilising DNA, they retard the development of colon tumours and tumour precursors (McIntosh *et al*., 1995). The anticarcinogenic activity of whey proteins and their individual components is well documented (Badger *et al*., 2001; McIntosh *et al*., 1995; Tsuda *et al*., 2000), and these proteins offer protection against colon and mammary cancers (Hakkak *et al*., 2000). α-La may also exert antiulcerative properties (Mezzaroba *et al*., 2006). Indeed, animal studies using rats demonstrated that α-la increases the gastric luminal pH and gastric fluid secretion and delays gastric emptying (Ushida *et al*., 2003). Serum albumin, α-la and lactoferrin are rich sources of the dipeptide γ-glutamylcysteine, which is an excellent source of dietary cysteine for cellular synthesis of glutathione, which protects against free radical damage, pollution, toxins and infection and contributes to defence mechanisms against cancer (Rogelj,

2000). An increase in cellular glutathione stimulates immunity, thus increasing antitumour effects in tumour cells (Bounous, 2000). Lactoferrin binds iron, which is potentially procarcinogenic thereby preventing intestinal damage. Indeed, the preventive effects of lactoferrin and lactoferricin on chemically induced colon carcinogenesis in rats and on transplanted carcinoma cell metastasis in mice have been demonstrated (Tsuda *et al*., 2006). Bovine serum albumin is another whey protein that may have anticancer properties, as it has been shown to inhibit growth of the human breast cancer (Laursen *et al*., 1990).

Milk contains substances that provide passive protection against infection in the intestinal lumen. This protection mainly involves immunoglobulins, lysozyme, lactoperoxidase and lactoferrin. Indeed, the antimicrobial activity of α-la and β-lg has been attributed mainly to peptides deriving from their enzymatic hydrolysis (Hartmann and Meisel, 2007; Madureira *et al*., 2007). Both intact α-la and β-lg do not present significant antibacterial activity (Pellegrini *et al*., 1999; Pihlanto-Leppala *et al*., 1999). The antiviral activity of both α-la and β-lg has also been reported by Chatterton *et al*. (2006). Immunoglobulins mainly consist of secretory IgA in human milk and IgG in cow milk. In particular, the main immunoglobulin present in cows' milk is IgG1 with lower amounts of IgM, IgA and IgG2, and they have been shown to partially resist degradation in the intestinal lumen (Roos *et al*., 1995). These proteins are responsible for protection against microbial pathogens, activation of complement, stimulation of phagocytosis, prevention of the adhesion of microbes and neutralisation of viruses and toxins (Mehra *et al*., 2006). They also increase the intracellular levels of glutathione, which is a key cellular antioxidant (McIntosh *et al*., 1998). Lactoferrin is a major protein in human whey, being present only at a low level in cow milk. The defence against a broad range of microbial infections has been historically ascribed to lactoferrin (Farnaud and Evans, 2003), and this defence depends on its concentration and on the degree of iron saturation. Some, if not all, of this activity results from lactoferricin, the bactericidal peptide formed during the peptic digestion of lactoferrin, whose antimicrobial activity originates from a direct interaction with the bacterial surface (Tomita *et al*., 1994). The antibacterial property of lactoferrin and lactoferricin protects against several bacterial strains and yeast (Tomita *et al*., 1994). Besides this action, lactoferrin and lactoferricin present anti-inflammatory, immunosuppressive or immunostimulatory activities, promoting lymphocyte proliferation in vitro (Tomita *et al*., 1994). Both lactoferrin and lactoferricin exhibit antiviral activity against hepatitis C (Iwasa *et al*., 2002; Kaito *et al*., 2007), human papillomavirus (Mistry *et al*., 2007) and herpes simplex virus (Jenssen, 2005). Recombinant human and bovine lactoferrins are now available for development into nutraceutical and pharmaceutical products (Weinberg, 2007).

Many cytokines and chemokines have been discovered in cows' milk, including interleukins, growth factors and chemokines that produce various effects, which contribute to the development and function of new tissues and of the immune system. In particular, several growth factors have been found in cows' milk at trace levels, including epidermal growth factor (EGF) and insulin growth factors 1 and 2 (IGF1, IGF2). They are very sensitive and easily destroyed by heating. Their effects on intestinal mucosal growth have been demonstrated, as shown by their ability to stimulate intestinal enzyme expression and maturation of intestinal function in newborns (Ma and Xu, 1997; Young *et al*., 1990). The highest amount of these proteins is found in colostrum during the first days of lactation. Colostral growth factors (IGF-1, EGF and others) in addition to the availability of dietary AA affect protein synthesis in neonates (Burrin *et al*., 1992), and, along with hormones (insulin, growth hormone and others), they contribute to the initiation of normal digestive function in newborn calves (Baumrucker *et al*., 1994; Hammon and Blum, 1998).

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