Chapter 6
α_2-Macroglobulins: Structure and Function

Irene Garcia-Ferrer, Aniebrys Marrero, F. Xavier Gomis-Rüth, and Theodoros Goulas

Abstract α_2-macroglobulins are broad-spectrum endopeptidase inhibitors, which have to date been characterised from metazoans (vertebrates and invertebrates) and Gram-negative bacteria. Their structural and biochemical properties reveal two related modes of action: the "Venus flytrap" and the "snap-trap" mechanisms. In both cases, peptidases trigger a massive conformational rearrangement of α_2-macroglobulin after cutting in a highly flexible bait region, which results in their entrapment. In some homologs, a second action takes place that involves a highly reactive β-cysteinyl-γ-glutamyl thioester bond, which covalently binds cleaving peptidases and thus contributes to the further stabilization of the enzyme:inhibitor complex. Trapped peptidases are still active, but have restricted access to their substrates due to steric hindrance. In this way, the human α_2-macroglobulin homolog regulates proteolysis in complex biological processes, such as nutrition, signalling, and tissue remodelling, but also defends the host organism against attacks by external toxins and other virulence factors during infection and envenomation. In parallel, it participates in several other biological functions by modifying the activity of

I. Garcia-Ferrer
Proteolysis Lab, Structural Biology Unit, "María de Maeztu" Unit of Excellence, Molecular Biology Institute of Barcelona (CSIC), Barcelona Science Park; c/Baldiri Reixac, 15-21, 08028 Barcelona, Spain

Present address: EMBL Grenoble, 71 Avenue des Martyrs; 38042 CS 90181, Grenoble Cedex 9, France
e-mail: irgarcia@embl.fr.

A. Marrero
Proteolysis Lab, Structural Biology Unit, "María de Maeztu" Unit of Excellence, Molecular Biology Institute of Barcelona (CSIC), Barcelona Science Park; c/Baldiri Reixac, 15-21, 08028 Barcelona, Spain

Present address: Department of Chemistry, University of Zürich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
e-mail: aniebrys.marrero@chem.uzh.ch.

F.X. Gomis-Rüth • T. Goulas (✉)
Proteolysis Lab, Structural Biology Unit, "María de Maeztu" Unit of Excellence, Molecular Biology Institute of Barcelona (CSIC), Barcelona Science Park; c/Baldiri Reixac, 15-21, 08028 Barcelona, Spain
e-mail: thgcri@ibmb.csic.es

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cytokines and regulating hormones, growth factors, lipid factors and other proteins, which has a great impact on physiology. Likewise, bacterial α_2-macroglobulins may participate in defence by protecting cell wall components from attacking peptidases, or in host-pathogen interactions through recognition of host peptidases and/or antimicrobial peptides. α_2-macroglobulins are more widespread than initially thought and exert multifunctional roles in both eukaryotes and prokaryotes, therefore, their on-going study is essential.

Keywords Macroglobulin • Proteinase inhibitor • Conformational change • Growth factor and cytokine regulator

6.1 Introduction

The ability of organisms to tightly control internal processes and protect themselves against antagonism or parasitism determines their success for well-being and reproduction. From single-cellular archaea to more complicated multicellular eukaryotes, and from invading parasites to defending hosts, a consortium of peptidases and their inhibitors are involved in the regulation of many physiological processes and in the protection against invasion and virulence (Armstrong 2006). Many peptidases and inhibitors have been characterised to date, forming a tightly balanced equilibrium.

α_2-Macroglobulins (α_2Ms) are the largest and among the most relevant inhibitors, due to their universal ability to counteract a broad spectrum of endopeptidases. These inhibitors are multi-domain proteins that use two related mechanisms to act as molecular traps instead of catalytic-centre blocks (Sottrup-Jensen 1989). Rather than simple inhibitors, they should be envisaged as sophisticated binding proteins that are activated by limited proteolysis and augment the clearance of binding proteins from the circulation. The liver is the principal site of synthesis in mammals and α_2Ms are found in blood, presumably to have better access to all parts of the body, where they contribute to the bulk of the innate immune system (Andus et al. 1983). Besides peptidase regulation, α_2Ms play a major role in controlling the action of many other effector molecules and modulating the physiology of the host, with many implications in health and disease (Borth 1992).

α_2Ms are anciently diverging molecules, which were initially described in eukaryotes, together with their homologs from the complement component family (Sottrup-Jensen et al. 1985). Human α_2M was first described back in 1946 (Cohn et al. 1946) as a major protein of blood serum, and bacterial orthologs were serendipitously discovered six decades later (Budd et al. 2004). Many years of intensive research has revealed several of their characteristics, which has resulted in a vast number of publications. Here, the unique structural and mechanistic features that enable α_2Ms to interact with a wide variety of peptidases and other physiologically relevant proteins are discussed.

6.2 Evolutionary Origin

α_2Ms belong to the thioester-containing proteins (TEPs) with members sharing a common evolutionary origin and conserved structural and functional features. Other TEPs are the peptidase inhibitors pregnancy zone protein (PZP) and α_1-inhibitor-3 (α_1I3), the complement components C3, C4 and C5, the cell surface antigen CD109, the complement C3 and PZP-like α_2M domain-containing 8 (CPAMD8), and insect and nematode TEPs (Nonaka 2011; Sottrup-Jensen et al. 1985). All these proteins undergo proteolytic processing and structural rearrangements as part of their role in the immune system of host defence. In most cases, they are characterized by the presence of an active thioester bond and have essentially the same domain organisation as the complement C3. However, in some members, the anaphylatoxin (ANA) domain is replaced with a bait region domain (BRD) or a hyper-variable region, and the C345C domain at their C-terminus is missing (Fig. 6.1) (Marrero et al. 2012; Janssen et al. 2005; Baxter et al. 2007; Garcia-Ferrer et al. 2015). Insect TEPs show sequence relatedness halfway between complement components and α_2M. However, like C3, they have a conserved histidine to convert the reactive glutamine/glutamate residue of the activated thioester to an intermediate form that favours its subsequent covalent linkage to hydroxyl groups rather than amines, as found in α_2Ms (Dodds et al. 1996; Law and Dodds 1997; Janssen et al. 2005; Baxter et al. 2007). In accordance with their structural differences, C3 and insect TEPs are functionally different from α_2Ms and are involved in bacterial opsonization, lysis or phagocytosis, rather than in peptidase trapping, inhibition and clearance (Sahu and Lambris 2001; Armstrong 2010). CD109 is a glycosyl-phosphatidyl-inositol-linked glycoprotein that was originally found in endothelial cells, platelets and activated T-cells with complement-like activity (Lin et al. 2002). CPAMD8 has a C-terminal Kazal-type serine peptidase inhibitor-like domain and is mainly expressed in kidney, brain and testis, with a poorly characterized function (Li et al. 2004).

Currently, all members of the TEP superfamily are sub-classified to the α_2M family, which includes α_2M, PZP, α_1I3, CD109 and CPAMD8, and the family of structural homologs of C3, which includes complement components C3, C4 and C5 (Sottrup-Jensen et al. 1985; Fujito et al. 2010; Baxter et al. 2007; Nonaka 2011). Insect TEPs could form a distinct family; however, phylogenetic analysis clearly indicates that they belong to the α_2Ms, which suggests that their functional similarity to mammalian C3 was caused by convergent molecular evolution (Nonaka 2011). The prevalence hypothesis, based on sequence similarity and exon-intron organization studies, states that all members of the TEP superfamily emerged from a common α_2M ancestor and underwent several gene duplication events. One of the duplicated α_2M genes was modified to form a C3/C4/C5 ancestral gene, which was then further duplicated to form the C4 and C3/C5 genes. Finally, the C3/C5 gene was duplicated to produce the C3 and C5 genes (Sahu and Lambris 2001; Nonaka 2011).

All α_2Ms have considerable importance in the innate immune response in metazoans, and emerged at least 600–700 million years ago, predating the emergence of

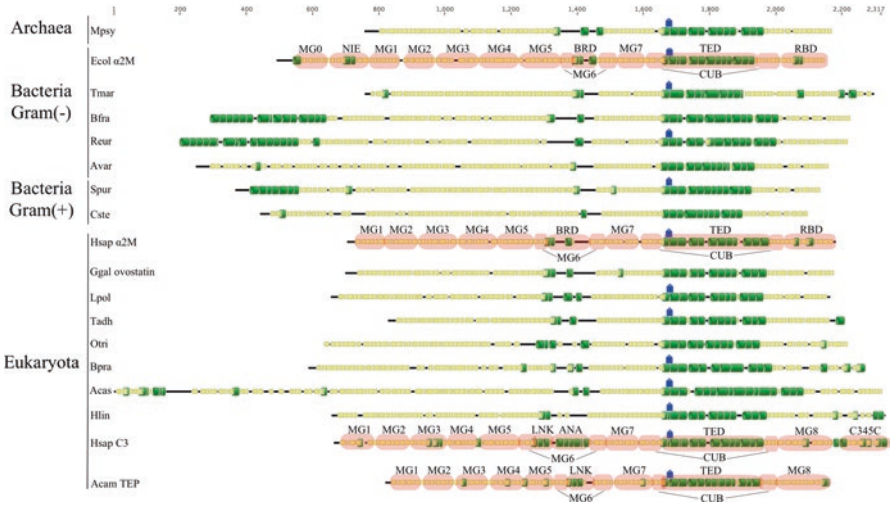

Fig. 6.1 Selected thioester-containing proteins (TEPs) from archaea, bacteria and eukaryota. The sequences are aligned based on the thioester bond motif (CXEQ) found in TED domains. No further alignment was performed due to the excessive phylogenetic distances. The secondary structure is based on existing structural models or on predictions by JPred (Drozdetskiy et al. 2015). β-strands are shown by *yellow bars*, α-helixes by *green barrels*, and the thioester bond by a *blue arrow*. The domains of homologs with known three-dimensional structures are further annotated with *red highlights*. MG, macroglobulin-like domains; BRD, bait region domain; CUB, C1r/C1s, Uegf, and Bmp1 found domain; TED, thioester domain; RBD, receptor-binding domain; LNK, linker domain; ANA, anaphylatoxin domain. Sequence names are shown with a four letter abbreviation, and further defined herein with their UniProt access number and a short description: Mpsy, *Methanolobus psychrophilus* (K4MDH4; Psychrophilic methanogen); Ecol α_2M, *Escherichia coli* α_2M (P76578; Gut habitant); Tmar, *Thermotoga maritima* (Q9X079; Extremophile); Bfra, *Bacteroides fragilis* (A0A0K6BRA4; Gut habitant); Reur, *Rhodopirellula europaea* (M2A507; Marine bacterium); Avar, *Anabaena variabilis* (Q3MAL1; Cyanobacterium); Spur, *Streptomyces purpurogeneiscleroticus* (A0A0M8ZE73; Soil habitant); Cste, *Clostridium stercorarium* (L7VLL5; Cellulolytic thermophile); Hsap α_2M, *Homo sapiens* α_2M (P01023); Ggal, *Gallus gallus* ovostatin (P20740; Egg white protein); Lpol, *Limulus polyphemus* (O01717; Ancient crab); Tadh, *Trichoplax adhaerens* (B3RVT5; Placozoa); Otri, *Oxytricha trifallax* (J9J1G5; Protozoa); Bpra, *Bathycoccus prasinos* (K8F745; Alga); Acas, *Acanthamoeba castellanii* (L8GY08; Amoebozoa); Hlin, *Haliplanella lineata* (D4QA01; Sea anemone); Hsap C3, *Homo sapiens* complement C3 (P01024); Agam TEP, *Anopheles gambiae* TEP (Q9GYW4; Mosquito)

immunoglobulins (Igs) (Sahu and Lambris 2001). Specifically, the most ancient member of the α_2M family described so far is from the hemolymph of the horseshoe crab, *Limulus polyphemus*, one of the oldest surviving multicellular organisms, which dates back over 500 million years (Enghild et al. 1990; Quigley et al. 1991; Rudloe 1979). This protein incorporates both glycerol and methylamine into the thioester, and could be considered a link between peptidase inhibitors and the complement components. Several other α_2Ms were biochemically characterized (Table 6.1 and references therein), and thousands have been identified in genome sequencing projects (Starkey and Barrett 1982; Budd et al. 2004). Accordingly, they are found in several eumetazoan phyla, including Cnidaria (e.g. sea anemone

Table 6.1 List of characterized α_2Ms

Name	Organism/UniProt no.	Localization	Mass (kDa)/Oligom./ Thioester			References
α_2M	*Homo sapiens*/P01023	Blood serum	720	Tetramer	+	Barrett et al. (1979), Marrero et al. (2012), and Sottrup-Jensen (1989)
α_2ML1	*Homo sapiens*/A8K2U0	Epidermis	180	Monomer	+	Galliano et al. (2006)
PZP	*Homo sapiens*/P20742	Pregnancy blood serum	720	Tetramer	+	Sand et al. (1985) and Sottrup-Jensen et al. (1984a)
Ovostatin	*Gallus gallus*/P20740	Egg white	780	Tetramer	–	Nagase and Harris (1983)
α_1M	Rabbit	Blood serum	~	Tetramer	+	Banbula et al. (2005)
α_2M	*Biomphalaria glabrata* (gastropod mollusc)	Hemolymph	800	Tetramer	+	Bender and Bayne (1996)
α_2M	*Ornithodoros moubata* (tick)	Plasma	420	Dimer	+	Kopacek et al. (2000)
α_1I3	*Rattus norvegicus*/P14046	Blood serum	174	Monomer	+	Enghild et al. (1989)
α_1M	*Rattus norvegicus*/Q63041	Blood serum	~	Tetramer	~	Xiao et al. (2000)
α_2M	*Limulus polyphemus* (horseshoe crab)	Hemolymph and blood cells	354	Dimer	+	Enghild et al. (1990), Armstrong et al. (1991), and Husted et al. (2002)
α_2M	*Erinaceus europaeus* (hedgehog)	Plasma	800	Tetramer	~	De Wit and Weström (1987)
$\alpha_2\beta$M	*Erinaceus europaeus* (hedgehog)	Plasma	450–550	Dimer	~	De Wit and Weström (1987)
α_2M	*Penaeus vannamei* (white shrimp)	Hemolymph	360	Dimer	+	Gollas-Galvan et al. (2003)
IrA2M	*Ixodes ricinus* (tick)	Hemolymph	440	Dimer	+	Buresova et al. (2009)
α_2M	*Chelonia mydas japonica* (turtle)	Blood serum	~	~	+	Ikai et al. (1988)
Ovostatin	*Chelonia mydas japonica* (turtle)	Egg white	~	~	–	Ikai et al. (1988)
αM	*Rana catesbiana* (frog)	Blood serum	180	Monomer	+	Rubenstein et al. (1993)
α_2M	*Astacus astacus* (crayfish)	Hemolymph	390	Dimer	+	Stöcker et al. (1991)

(continued)

Table 6.1 (continued)

Name	Organism/UniProt no.	Localization	Mass (kDa)/Oligom./ Thioester			References
α_2M	*Octopus vulgaris* (mollusc)	Hemolymph	360	Dimer	+	Thøgersen et al. (1992)
α_2M	*Struthio camelus* (ostrich)	Blood serum	779	Tetramer	+	Van Jaarsveld et al. (1994)
α_2M	*Helix pomatia* (gastropod mollusc)	Hemolymph	697	Tetramer	+	Yigzaw et al. (2001)
α_2M	*Pacifastacus leniusculus* (crayfish)	Hemolymph	380	Dimer	+	Hergenhahn et al. (1988)
α_2M	*Libinia emarginata* and *Cancer borealis* (crab)	Hemolymph	480–460	Dimer	+/−	Armstrong et al. (1985)
α_2M	*Homarus americanus* (lobster)	Hemolymph	342	Dimer	+	Spycher et al. (1987)
α_2M	*Cyprinus carpio* (bony fish carp)	Blood serum	380	Dimer	+	Mutsuro et al. (2000)
α_2M	*Farfantepenaeus paulensis* (shrimp)	Blood serum	389	Dimer	+	Perazzolo et al. (2011)
ECAM	*Escherichia coli*/P76578	Inner membrane lipoprotein	183	Monomer	+	Doan and Gettins (2008), Neves et al. (2012), Garcia-Ferrer et al. (2015), and Fyfe et al. (2015)
Sa-α_2M	*Salmonella enterica* ser. Typhimurium/Q8ZN46	Inner membrane lipoprotein	179	Monomer	+	Wong and Dessen (2014)
MagD (YfaS)	*Pseudomonas aeruginosa*/PA4489	Inner membrane lipoprotein	165	Monomer	−	Robert-Genthon et al. (2013)

Where: (+) presence, (−) absence, and (~) unknown

Haliplanella lineata), Protostomia (e.g. Ecdysozoa: Arthropoda and Nematoda; Lophotrochozoa: Mollusca and Annelida), Deuterostomia (Echinozoa and Chordata) and Platyhelminthes (e.g. *Schistosoma haematobium*) (Fujito et al. 2010; Nonaka 2011). In addition, some more recent initial studies have demonstrated that bacterial α_2Ms are found almost exclusively in free-living, symbiotic and pathogenic Gram-negative bacteria, including proteobacteria, fusobacteria, spirochetes, bacteroidetes, deinococcus, cyanobacteria, planctomycetes and thermotogae (Budd et al. 2004). They were probably acquired one or more times from metazoan hosts and were then spread widely through other colonizing bacterial species by more than ten independent horizontal gene transfer events. However, this is not definite, particularly bearing in mind the putative presence of α_2Ms in free-living *Thermotoga maritima* and archaea species (e.g. *Methanolobus psychrophilus*) and the evidence of lateral gene

transfer events through their symbiosis (Nelson et al. 1999; Budd et al. 2004; Wong and Dessen 2014). Considerable evidence of putative TEPs can also be identified in protozoa, such as Amoebozoa (e.g. *Acanthamoeba castellanii*), Alveolata (e.g. *Oxytricha trifallax*), green algae Viridiplantae (e.g. *Bathycoccus prasinos*) and in some Gram-positive bacteria (e.g. *Clostridium stercorarium*, *Halothermothrix orenii* and *Streptomyces purpurogeneiscleroticus*) (Fig. 6.1). All these proteins share an overall similar secondary structure and carry a consensus sequence for a thioester bond; however, more detailed investigation are required to reveal their extent and evolutionary relationship, and to assign them a biological role.

6.3 Structural Aspects of α_2Ms

6.3.1 Primary Structure, Sequence Motifs and Post-translational Modifications

α_2Ms are large macromolecules with 1474 amino acid residues in the human homolog, 1653 in *Escherichia coli*, and over 2000 in other putative sequences (Fig. 6.1) (Doan and Gettins 2008; Sottrup-Jensen et al. 1984b). Close inspection of the sequences reveals several motifs that are important for protein functionality. Among the most important and highly conserved is the motif that encodes the formation of the hyperactive thioester bond (C-X-E-Q: X for G or L) (Sottrup-Jensen et al. 1985). It is present in almost all the TEPs that have been characterized to date and will be further discussed below.

All primary structures start with a 17- (*E. coli* α_2M; hereafter ECAM) to 40-residue (chicken egg-white α_2M; hereafter ovostatin) peptide that is required for signalling protein translocation, either to the blood serum and hemolymph in vertebrates and invertebrates, or to the periplasm in Gram-negative bacteria (Nielsen et al. 1994; Doan and Gettins 2007; Sottrup-Jensen 1989). In some bacterial homologs, a "lipobox" consensus motif succeeds the signal peptide (in ECAM: L-A-G|C-D), which contains a cysteine that will become the N-terminus of the mature protein after post-translational addition of a diacylglycerol moiety to the side chain of the cysteine, and of a palmitoyl group through amide linkage to the N-terminus. In addition, the presence of an aspartic acid at position two, following the cysteine, indicates that the mature lipoprotein should be retained by the inner membrane, and is therefore localized in the periplasmic space (Doan and Gettins 2008; Pugsley 1993; Seydel et al. 1999).

In contrast to the bacterial homologs, eukaryotic α_2Ms are stabilized by an extensive network of disulphide bonds (Doan and Gettins 2008; Robert-Genthon et al. 2013; Wong and Dessen 2014). Tetrameric human α_2M and ovostatin contain twelve intra-chain disulphide bonds and two inter-chain bonds between adjacent monomers (Jensen and Sottrup-Jensen 1986; Marrero et al. 2012; Sottrup-Jensen et al. 1984b; Nielsen et al. 1994). Intra-chain disulphides are also present in monomeric

α_2ML1 from epidermis, and dimeric α_2Ms from *L. polyphemus*, which is further stabilized by an inter-chain bond between dimers (Galliano et al. 2006; Iwaki et al. 1996; Husted et al. 2002).

Extensive glycosylation is also found in eukaryotic homologs, with ovostatin binding 56 glucosamine molecules per subunit, with 12 of its glycosylation motifs (N-X-S/T) likely to be glycan-linked (Nielsen et al. 1994). Human α_2M has a total of 32 glycosylation sites per tetramer, with an average of 31 glycan-chains attached per molecule. These consist of mannose, galactose, N-acetyl-glucosamine, sialic acid and fucose residues (Dunn and Spiro 1967b). In *L. polyphemus*, out of seven potential glycosylation sites, six carry common glucosamine-based carbohydrate groups, whereas one carries a carbohydrate chain containing both glucosamine and galactosamine (Husted et al. 2002). Glycosides generally protrude from the surface of the structures, where they contribute to protein solubility and stability, and participate in interactions with other molecules (Marrero et al. 2012; Sottrup-Jensen et al. 1984b; Dunn and Spiro 1967a, b; Arnold et al. 2006; Goulas et al. 2014; Paiva et al. 2010). In bacterial α_2Ms, glycosylation has not been reported so far and is not expected, since this modification is almost exclusively restricted to eukaryotic organisms, with the exception of some Gram-negative bacteria, such as *Campylobacter jejuni* (Nothaft and Szymanski 2010; Wacker et al. 2002).

6.3.2 Secondary Structure Conservation and Domain Organization

The first structural details at atomic resolution were derived from individual α_2M domains of human and bovine origin, and from full-length models of their close homologs found in complement components (Doan and Gettins 2007; Jenner et al. 1998; Janssen et al. 2005; Huang et al. 2000). Human α_2M consists of 11 domains, corresponding to five structurally different moieties (Fig. 6.1) (domain terminology based on (Marrero et al. 2012; Garcia-Ferrer et al. 2015). The first seven domains, termed macroglobulin-like (MG) domains, MG1-MG7, are seven-stranded antiparallel β-sandwiches comprising a three- and a four-stranded sheet, and are arranged as a central scaffold, into which additional elements are inserted. These insertions result in domains that vary in length, spanning approximately 70 to 128 residues. Exceptionally, in bacterial homologs, there are two extra domains at the N-terminal, an MG0 and a NIE domain (from N-terminal domain of induced ECAM), with the former being a variant of MG domains with an extra inserted short β-strand (Garcia-Ferrer et al. 2015; Wong and Dessen 2014; Fyfe et al. 2015). In domain MG6, an irregularly folded bait-region domain (BRD) is inserted (66 to 126 residues). The BRD is stabilized by three helices, as well as through interactions with adjacent MG domains and the C-terminal receptor-binding domain (RBD). BRD is in an extended conformation and freely accessible to endopeptidases. Following MG7, an all-β CUB domain (C1r/C1s, Uegf, and Bmp1 found domain) is found adjacent to MG6 and it consists of two four-stranded antiparallel β-sheets unrelated to the MG fold.

Inserted within and placed below the CUB domain, the thioester domain (TED) features a ~310-residue helical domain with an α/α-toroid topology. It consists of six concentric α-hairpins, arranged as a sixfold α-propeller around a central axis. This architecture gives rise to a thick disc with two parallel flat sides, the entry and the exit side, which are shaped by the N- and C-termini of the ring of inner helices. In the native state of α_2M, a buried thioester bond is formed between the side chains of the cysteine and the glutamine/glutamate residues within the TED segment (C-G-E-Q). After TED, the polypeptide chain rejoins CUB, which leads to the C-terminal domain, RBD, also known as MG8. RBD features the third type of β-sandwich architecture found within α_2Ms, and forms a variant of the MG fold into which a β-α-β motif is inserted, thus giving rise to a four-stranded and a five-stranded twisted sheet. In the isolated structures of human and bovine RBD, a calcium-binding site is present in the loop region at one end of the β-sandwich (Huang et al. 2000; Jenner et al. 1998). Calcium binding principally affects this loop region and does not significantly perturb the stable core structure of the domain.

6.3.3 Tertiary Structure and Conformational Changes During Induction

6.3.3.1 Early Studies

Determination of the α_2M structure proved a long process, due to difficulties arising from sample heterogeneity owing to glycosylation, but also from the large size and intrinsic flexibility of the molecule (Andersen et al. 1991, 1994; Dolmer et al. 1995; Goulas et al. 2014). The first low resolution models appeared in 1968 and were obtained by negative-staining and cryo-electron microscopy (cryo-EM) (Andersen et al. 1995; Bloth et al. 1968). These models described human α_2M as graceful monograms of the letters "H", "I" and the Russian letter "Ж", which succeeded the preceding denominations "lip", "padlock", "cross", "four-petaled flower", and "eye" (Larquet et al. 1994). Technological advances and the combination of techniques, such as cryo-EM, combined with image processing using three-dimensional reconstructions and low-resolution X-ray models, provided a more accurate view of the markedly different shapes of the molecule, both in the native and induced forms. This contributed to rational and conclusive explanations of the mechanism of action (Kolodziej et al. 2002; Marrero et al. 2012; Garcia-Ferrer et al. 2015).

6.3.3.2 From Tetramers to Monomers and Vice-Versa

The α_2M orthologs studied to date have a molecular mass under denaturing conditions of 150–200 kDa, which gives rise in the native state to monomeric, dimeric or tetrameric arrangements of 165–800 kDa (Table 6.1) (Armstrong et al. 1991; Barrett et al. 1979; Doan and Gettins 2008; Enghild et al. 1989). Like most eukaryotic

homologs, human α_2M is a homotetramer (Fig. 6.2C) made up of the non-covalent association of two dimers, a top and a bottom dimer, in an antiparallel or head-to-tail orientation. Each dimer consists of two monomers covalently linked by two symmetric inter-chain disulphide bonds. This structure was initially proposed from sequencing studies of human α_2M showing that the two subunits are linked in an antiparallel orientation by two disulphide bonds close to their N-termini, and subsequently validated by antibody-labelled cryo-EM models and X-ray structures (Kolodziej et al. 1996; Marrero et al. 2012; Qazi et al. 2000; Jensen and Sottrup-Jensen 1986). The tetramers may dissociate into dimers, either after limited reduction of the intra-chain disulphide bonds or by pH or salt-induced dissociation, but they maintain their overall architecture and the labile thioester bond unreacted. The dimers or even monomers are active towards peptidases and resemble the characteristics of the monomeric and dimeric homologs (Armstrong et al. 1991; Doan and Gettins 2007; Gonias and Pizzo 1983; Pochon et al. 1989; Shanbhag et al. 1996). However, reaction with peptidases or chemical induction renders the monomers or dimers prone to di- or tetramerisation, which relates with the observation that induced tetramers are more difficult to dissociate and tightly engaged (Barrett et al. 1979). Monomeric α_2Ms, including the bacterial homologs and rat α_1I3, act similarly by forming stable elongated dimers after induction, but only by specific peptidases (Fig. 6.2F, G) (Enghild et al. 1989; Garcia-Ferrer et al. 2015; Gonias and Pizzo 1983). Besides bait cleavage, in the bacterial homolog ECAM a second proteolytic cleavage at the interface between the MG0 and NIE domain is required before dimer formation (Garcia-Ferrer et al. 2015).

6.3.3.3 Conserved Multi-domain Structure

Superposition of induced monomers from both bacterial and human α_2Ms reveals that they conserve the overall shape (Fig. 6.2A, B). A detailed inspection, however, indicates that only a core consisting of domains MG2, MG3, MG6 and MG7 can be reasonably well-matched (Garcia-Ferrer et al. 2015). The first six MG domains (MG1-MG6) are arranged as a compact ellipsoidal one and a half-turn right-handed superhelix resembling a distorted “key ring”. Preceding this arrangement, two structurally flexible domains (MG0 and NIE) protrude in bacterial homologs, and possibly act as a membrane anchor for cell wall attachment. Inserted within MG6, BRD is framed by the key ring scaffold (Fig. 6.2D) and lines most of the vertical dimension of the formed cavity. It is stabilized by several interactions with MG, RBD and CUB domains, and partly defined in the Fourier density map, which indicates conserved intrinsic flexibility (Marrero et al. 2012; Wong and Dessen 2014; Garcia-Ferrer et al. 2015).

In induced α_2Ms, perpendicularly attached to MG3 and MG6, MG7 closes the MG-superhelix like a plug and forms a protruding structure in the upper limit of the molecule, which was initially attributed to the RBD domains (Qazi et al. 1999; Kolodziej et al. 2002; Marrero et al. 2012). MG7 leads to the hook of the molecule that includes the CUB, TED and RBD domains. The TED domain, inserted into

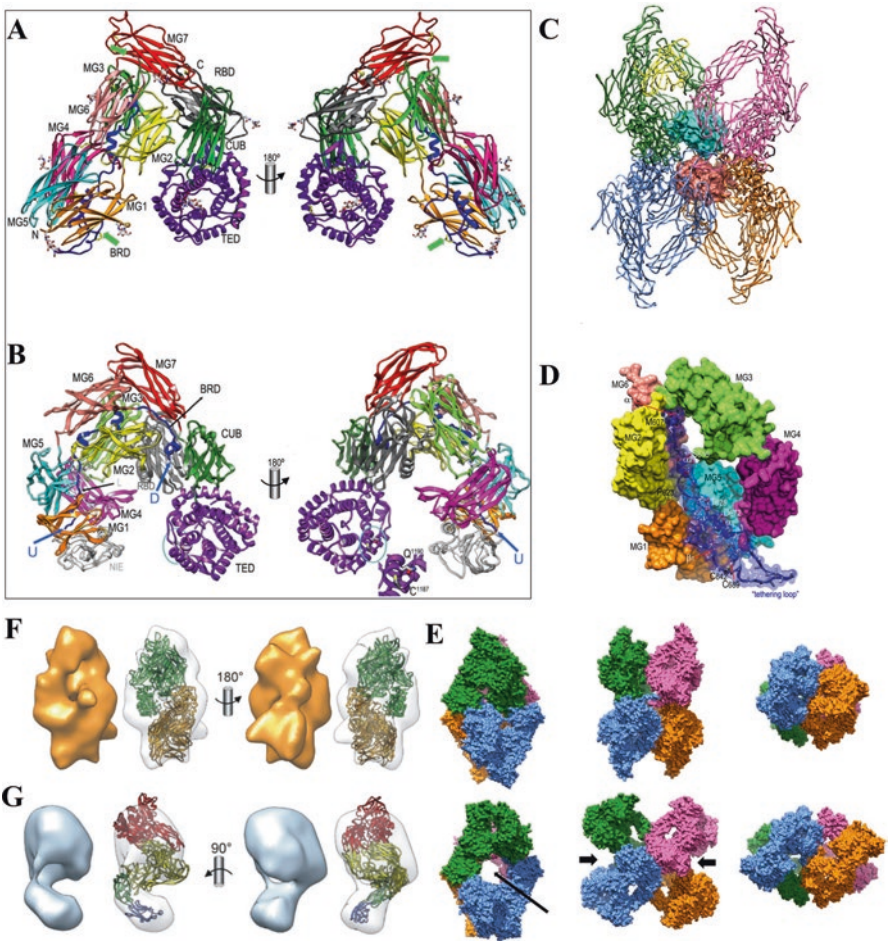

Fig. 6.2 Three-dimensional experimental structures of human and *E. coli* α_2-macroglobulin (hα_2M and ECAM, respectively). (**A**) Ribbon plot of a methylamine-induced hα_2M monomer in front view (convex face; left) and back view (concave face; right). *Green arrows* pinpoint the anchor points of the flexible bait region. (**B**) Ribbon plot of monomeric-induced ECAM in front and back views. The domain colours are as in A, and the visible polypeptide chain ends upstream (U) and downstream (D) of the cleaved bait region, as well as the thioester (*cyan ellipse*). (**C**) Possible representation of the hα_2M in H-view as a coil with two trapped molecules of HIV-1 peptidase tentatively modelled as *cyan* and *salmon pink solid surfaces*. Each monomer is represented in a separate colour. The RBD, which is only defined for monomer D, is further shown in *yellow*. (**D**) Detail of the methylamine-induced hα_2M structure depicting domains MG1-MG6 as rendered surfaces coloured according to A, and BRD as a *dark-blue ribbon* with *grey* side chains – some of which were truncated after C – superposed with its semi-transparent Connolly surface. Secondary structure elements of BRD are labelled, as are the tethering loop and selected residues for reference. (**E**) X-view, H-view and End-view of the experimentally induced hα_2M (*upper row*) and the native tentatively modelled tetramer (*lower row*). *Black arrows* pinpoint the large entrance to the central cavity. (**F**, **G**) Dimeric-induced ECAM and monomeric native ECAM single-particle cryo-EM reconstructions. The cryo-EM maps (*solid surface*) and the fitting of the respective atomic models (*semi-transparent surfaces*) are shown in two views (Figures are reproduced with permission from Garcia-Ferrer et al. 2015; Marrero et al. 2012)

CUB, in its native conformation, maintains its thioester bond buried against RBD, but also interacts with MG2 and CUB domains. The C-terminal domain, RBD, occupies a key position in the human α_2M structure and based on cryo-EM models of antibody-labelled native protein, it is buried between the ends of two dimers and forms a chisel-like feature (Qazi et al. 1999; Kolodziej et al. 2002). In the induced form, RBD interacts with TED and CUB domains, but is rather flexible and detected only in one of the four monomers of the tetramer (Fig. 6.2C). In bacterial homologs, it further interacts with the MG7 domain and after activation, it stabilizes the hook structure protruding from the MG-superhelix through contacts with domains MG2 and MG3 (Garcia-Ferrer et al. 2015; Wong and Dessen 2014; Marrero et al. 2012).

6.3.3.4 Apertures and Prey-Trapping Chambers

Access of peptidases to the bait region varies in monomeric and tetrameric forms. In monomers, the bait protrudes from the "key ring" scaffold, and is freely accessible to the bulk solvent and to peptidases (Garcia-Ferrer et al. 2015; Wong and Dessen 2014). In tetramers, initial hypotheses suggested that it is also externally oriented due to the limited access of the peptidases through the narrow openings of the central cavities (Kolodziej et al. 2002). However, biochemical and more detailed structural information showed that the bait resides within the tetramer (Marrero et al. 2012; Bowen and Gettins 1998). Modelling studies suggested that peptidases possibly enter native α_2M through two rather circular apertures of ~40 Å, at the interface between vicinal monomers on opposite sides of the tetramer, which may permit access to molecules of at least 20–25 kDa (Fig. 6.2E). These apertures would be framed symmetrically by MG1, MG2, and TED of each vicinal monomer and would be independently accessible, which explains how two peptidases can be caught and why small proteins like insulin or other nucleophiles can be bound during the process of complex formation (Marrero et al. 2012). Moreover, the propensity of native α_2M to dissociate into dimers at low protein concentration (10 μg/ml) or under particular biochemical conditions suggests that connectivity between the monomers is minimal, with the apertures probably being more relaxed and able to enlarge to permit better access to molecules (Boisset et al. 1996; Kolodziej et al. 2002).

After activation, several apertures are still present but with restricted size. In human α_2M, twelve entrances are found with some of them modulating the entrance from the bulk solvent by glycan chains acting like plugs (Schroeter et al. 1992; Marrero et al. 2012). The maximum dimensions of the apertures match the estimated size that would prevent non-covalently bound prey from escaping rapidly, whilst allowing small protein substrates or inhibitors to enter the central cavity (25–30 Å). In a similar manner, when monomeric α_2Ms dimerize after activation, a number of much larger entrances are created, which in turn explains why the ECAM protects only very high-molecular-mass proteins from digestion (Garcia-Ferrer et al. 2015; Doan and Gettins 2008).

In tetrameric α_2Ms, internalized peptidases are hosted in a central part of around 60 Å in diameter, termed the "prey chamber", which is delimited at the top and bottom by the segments of MG3 and MG4 that participate in disulphide-mediated dimerization (Marrero et al. 2012). The prey chamber is restricted at its centre, at half particle height, to a diameter of approximately 30 Å by loops provided by the four TEDs (the "narrowing belt"), and complemented by additional elongated volumes provided by the concave face of each of the four monomers ("substrate antechambers"). Overall, the prey chamber has space for up to two peptidases of 20–30 kDa, one above and one below the narrowing belt, that is, one for each disulphide-linked dimer (Fig. 6.2C). This is consistent with the maximal 2:1 stoichiometry of inhibition determined for peptidase binding by tetrameric α_2Ms (Barrett and Starkey 1973). Based on its size, the chamber could also accommodate a single, larger molecule of up to around 80–90 kDa, if the loops of the cavity-narrowing belt were folded back towards the inner wall of the cavity. In a similar manner, monomeric ECAM forms a central prey chamber of ~40 Å in diameter during dimerization, which may accommodate a single small-to-medium sized endopeptidase at best (Garcia-Ferrer et al. 2015).

6.3.3.5 Mechanisms of Peptidase Entrapment

Monomeric and tetrameric α_2Ms employ two approaches for trapping peptidases, named respectively as "the snap trap" and "Venus flytrap" mechanisms (Garcia-Ferrer et al. 2015; Marrero et al. 2012). In both cases, the principal trigger of activation is the peptidase attack on the bait region, which results in massive conformational change from the native to the induced form. This change can also be triggered by small amine molecules after direct reaction with the thioester (Barrett et al. 1979; Doan and Gettins 2008), but are restricted only in homologs with an active thioester bond and structural characteristics that permit the transition without bait cleavage (Garcia-Ferrer et al. 2015; Wong and Dessen 2014). Several structural studies of native (Kolodziej et al. 1996; Wong and Dessen 2014), methylamine- (Marrero et al. 2012; Qazi et al. 1999), trypsin-, chymotrypsin-, and plasmin-transformed α_2M (Kolodziej et al. 1998; Garcia-Ferrer et al. 2015) show how variations in the shape of the native and induced molecules are related to peptidase entrapment (Kolodziej et al. 2002).

Overall, tetrameric native α_2Ms are larger and broader than the induced molecules, with the conformational change causing strong lateral compression and vertical stretching of the native particle (Fig. 6.2E). This extraordinary change in the architecture functions to completely engulf one to two molecules of peptidases within the central cavity and irreversibly encapsulate them (Kolodziej et al. 2002). At the same time, the thioester bond is exposed to the solvent and rapidly reacts with exposed lysines extended from the surface of the peptidase. Trapped peptidases are still active, but unable to escape due to steric hindrance formed by the chambers or the covalent crosslinks with α_2M. The mechanism is so efficient that lack of thioester, as in ovostatin, does not compromise peptidase entrapment (Nagase and Harris 1983).

In the snap-trap mechanism, the conformational rearrangement in monomeric α_2M serves mainly to expose the thioester bond to the solvent and react with the attacking peptidase (Garcia-Ferrer et al. 2015; Enghild et al. 1989; Doan and Gettins 2008). In this way, α_2M nails peptidases, which thereafter are trapped and can no longer act freely. Also, the overall structure is more compact, and in some cases leads to dimerization, but without any obvious influence on inhibitory function. The efficiency of this mechanism relies only on the thioester's ability to act fast and crosslink attacking peptidases, and therefore, is much less efficient inhibitor than the tetrameric homologs.

6.3.4 Bait Region and Bait-Region Domain

The function of the BRD is to orient and expose the bait region in an extended conformation, so that attacking peptidases can easily approach and cut the chain (Marrero et al. 2012; Garcia-Ferrer et al. 2015). It is also the trigger for a massive conformational change, which leads to the entrapment of the attacking peptidase by α_2M. Changes in amino acid residues in the bait region can render molecules functional, but these changes might alter the specificity against non-cutting peptidases, such as tobacco-etch-virus peptidase (Van Rompaey et al. 1995; Garcia-Ferrer et al. 2015; Ikai et al. 1999). More radical modifications, such as removal of the entire BRD, give rise to α_2M with lack of functionality and greater propensity to digestion (Garcia-Ferrer et al. 2015; Gettins et al. 1995).

Typically, the bait region includes target peptidic bonds that are susceptible to attack by several endopeptidases, thus making α_2Ms ideal scavengers for the diverse array of enzymes produced by parasites or host that might attack during the lifetime of any given organism (Sottrup-Jensen et al. 1989; Sottrup-Jensen 1989). However, close inspection of the amino acid sequence shows limited conservation even in phylogenetically close species. Also, the eukaryotic bait domains and regions are much larger than the bacterial ones suggesting that this stretch may be under evolutionary pressure to provide reactivity to destructive peptidases of endogenous and exogenous origin, but restricts reactivity to essential endogenous peptidases of the tissue fluids (Garcia-Ferrer et al. 2015; Armstrong 2006).

6.3.5 The Reactive β-Cysteinyl γ-Glutamyl Thioester Bond

The thioester segment is a 15-atom thio-lactone ring composed of four residues (C-X-E-Q), located at the beginning of the first toroid helix of the TED on the domain entry face (Budd et al. 2004; Sottrup-Jensen 1989, 1994). The thiol-esterified glutamine/ate residue is encoded as glutamine, and the bond may be formed in reactions reminiscent of those of transglutaminases (Hall and Söderhall 1994). The intervening X (glycine or leucine) and glutamate residues are widely

conserved across thioester-containing proteins (Armstrong and Quigley 1999; Doan and Gettins 2008).

Similar to the structures of complement and insect TEPs, in α_2M the thioester is stable against hydrolysis, even though it is near to the surface of the molecule. The surrounding aromatic and hydrophobic residues arising from the TED and RBD domains form a hydrophobic pocket that is conserved in bacterial and eukaryotic variants (Baxter et al. 2007; Janssen et al. 2005; Garcia-Ferrer et al. 2015; Marrero et al. 2012). Removal of the RBD domain, or even single mutation of those residues, results in a properly folded α_2M, but with an open thioester bond as was also observed with complement C3 (Janssen et al. 2005; Wong and Dessen 2014). In contrast, the proceeding domains from MG7 do not play any role in the formation and stability of the thioester bond at least in bacterial species, so expression of α_2M fragments, from MG7 to the RBD domain, results in a well-folded protein with a formed thioester bond (Garcia-Ferrer et al. 2015).

An asparagine residue in human α_2M (N1088) was also found to be important. It plays a multifunctional role from the formation of the thioester bond to its subsequent reaction with nucleophiles and the conformational change induced by hydrolysis (Suda et al. 1997). Hydrolysis most likely proceeds *via* direct attack of a nucleophile on the thioester. Nucleophiles can be lysines protruding from the surface of attacking peptidases, or small amines like methylamine, ethylamine and ammonia (Barrett et al. 1979). In contrast, in complement and insect TEPs, this residue is a histidine that participates in the hydrolysis of the thioester bond *via* an acylimidazole intermediate and leads to covalent crosslinks with hydroxyls (Fredslund et al. 2006; Janssen et al. 2005; Suda et al. 1997). In some cases, as in *L. polyphemus*, instead of reacting with peptidases, the thioester crosslinks the opposite chains of α_2M and does not encage the entrapped peptidase molecule, so binding is again entirely non-covalent (Dolmer et al. 1996; Quigley et al. 1991).

6.3.6 Receptor Recognition and Endocytosis

After induction by peptidases, human α_2M exposes the C-terminal RBD domain, which is recognized by the ligand-binding domains of low-density lipoprotein receptor-related protein (LRP1 or CD91). This is a 600 kDa endocytic membrane-bound receptor, an essential member of the low density lipoprotein receptor family. It is expressed in a broad spectrum of cell types as a single-chain precursor, which is processed into a 85 kDa transmembrane β-chain and an approximately 515 kDa α-chain, non-covalently associated with the extracellular part of the β-chain (Lillis et al. 2008). The α-chain contains four clusters of 2, 8, 10, and 11 ligand-binding domains, known as complement-like repeats (CR), flanked by epidermal-growth-factor-type repeats, and β-propeller modules (Andersen et al. 2000). A functional CR consists of approximately 40 residues, with three conserved disulphide linkages, and it coordinates one calcium ion (Daly et al. 1995). The rest of the amino acids are variable, yielding CR domains that can bind more than 30 ligands of

different sizes and structures in addition to α_2M. They can all be replaced by receptor-associated protein (RAP), thus making LRP a multifunctional protein (Lillis et al. 2008).

α_2M binds with high-affinity to cluster 2 of LRP1, which contains eight CRs, and especially with CR3-4-5 domains rather than CR5-6-7 or the single repeats individually (Dolmer and Gettins 2006). Structural models of RBD in complex with CRs are not available, but biochemical studies evince that they are formed through interactions accomplished *via* a combination of charge-charge or polar contacts, together with significant hydrophobic interactionism in a similar manner as for RAP/CR complexes (Jensen et al. 2006). RBD exposes lysines (K1393 and K1397) as well as upstream hydrophobic residues, yielding a conserved motif (ΨKXΨK; Ψ is hydrophobic and X any amino acid residue) (Jensen et al. 2006; Nielsen et al. 1996). Mutation of the lysines or lack of them, as in chicken homolog ovostatin, is deleterious and results in complete failure of complex formation (Nielsen et al. 1996). In bacterial homologs, besides the overall structural similarity of RBD with the human ones, the α-helices are distorted and do not expose any lysine, thus lacking any possibility for interaction with LRP1 (Garcia-Ferrer et al. 2015).

Ligands bound extracellularly by LRP1 at neutral pH are rapidly internalized (the half-life of the complexes is 2–5 min) and then released in the endosomes (pH 6), leading to their subsequent lysosomal degradation. Dissociation of ligands is crucial for receptor recycling and proper function (Lillis et al. 2008). Complexes that are formed *in vivo* are not influenced by pH variations, therefore they would remain uninfluenced during the process of endocytosis. There is a mechanism of dissociation at acidic pH that involves the protonation of a histidine residue, which serves as a switch, allowing tighter binding of the neighbouring β-propeller domain than the binding ligands, which consequently leads to LRP1 discharging from bound molecules (Dolmer and Gettins 2006; Rudenko et al. 2002).

6.4 Role of α_2Ms in the Control of Proteolytic Activity

The best characterized function of the α_2M homologs is peptidase neutralization by a three-step process involving binding, inhibition and marking for endocytosis and intracellular degradation (Armstrong 2010). This process resembles the opsonization found in the homologous complement components and insect TEPs, which are covalently bound to the surfaces of foreign cells and marked for immune destruction (Le et al. 2012; Janssen et al. 2005). α_2M is an abundant protein in human plasma at concentrations ranging from 2 to 4 mg/ml, and is the second most abundant protein in the hemolymph of the cephalopod sepia, and the third most abundant in *L. polyphemus* (Enghild et al. 1990; Vanhoorelbeke et al. 1993; Sottrup-Jensen 1989). The concentration may vary with age and acute phase responses such as trauma, infection and inflammation, with more than one homolog potentially present in each organism and in the same or different organs. In humans, different α_2Ms

can be found in blood serum, epidermis and pregnancy serum (Sand et al. 1985; Galliano et al. 2006; Barrett et al. 1979; Tunstall et al. 1975; Cray et al. 2009). In bacteria, and more precisely in *E. coli*, threefold overexpression of ECAM has been observed in the membranes under anaerobic conditions, but there is no further information available (Brokx et al. 2004).

In contrast to most inhibitors that selectively inhibit peptidases of a specific catalytic mechanism, α_2M targets all peptidases, regardless of the catalytic class or family (serine, cysteine, metallo, aspartic) (Kantyka et al. 2010; Hibbetts et al. 1999). In this case, specificity is characterized mainly by the accesses that α_2Ms assign to the peptidases to approach and cut the bait region, and to initiate the process of entrapment. As mentioned above (in the section Apertures and prey-trapping chambers), peptidases access the bait region of tetrameric α_2M through entrances of limited size. In some instances, they are too large to fit and to enter the cavity where the bait is positioned, and they fail to react with α_2M, as the case of the collagenase of *Clostridium perfringens* that has a molecular mass larger than 100 kDa (Abe et al. 1989). Moreover, the amino acid sequence of the bait region plays a central role in the universality of α_2Ms, and several peptidases fail to cut within it due to lack of the necessary sequences (e.g. the lysyl-specific endopeptidase from *Porphyromonas gingivalis* Kgp and *Achromobacter lyticus*) (Ikai et al. 1999; Gron et al. 1997). Non-reacting peptidases confer a type of immunity towards α_2M that can be of vital importance to the physiology of the organisms. For example, the clotting enzymes of *L. polyphemus* are not inhibited by endogenous α_2M. This is of physiological importance during blood clotting (Armstrong et al. 1984). However, the more exposed bait region of the monomeric and dimeric homologs broadens their inhibitory range towards peptidases of variable size. In this case, peptidase immunity is based on the presence of a well-positioned lysine residue on the surface that can be easily crosslinked by the thioester bond. In addition, the more open character of these α_2Ms permits the peptidases to have better access to larger peptides than in the case of tetramers, where they have to pass through narrow entrances to be trapped (Garcia-Ferrer et al. 2015; Marrero et al. 2012; Galliano et al. 2006; Enghild et al. 1989).

α_2Ms control several essential proteolytic events that take place in all living organisms ranging from digestion of complex molecules for the assimilation and physiological turnover of proteins, to activation of peptide hormones and effector proteins secreted as zymogens, blood clotting and clot resolution, inflammation and remodelling of the extracellular matrix (Lopez-Otin and Bond 2008). However, under some circumstances, endogenous peptidases have the potential for undesirable destructive action, and they should not survive to act beyond the limits of their intended function. The primary aim of human α_2M is rapid inhibition of excess peptidases released during tissue injury, such as those liberated by neutrophils at the site of inflammation. During phagocytosis and neutrophil turnover, neutrophil elastase, proteinase 3 and cathepsin G are released into the extracellular space as active peptidases, but tightly regulated in the extracellular and pericellular space to avoid degradation of connective tissue proteins including elastin, collagen, and proteoglycans (Korkmaz et al. 2010). Human chymase, a chymotrypsin-like peptidase, activates

many important biological mediators, such as angiotensin, interleukin-1-β (IL-1-β), big endothelin, and interstitial collagenase with a potential role in hypertension and atherosclerosis. It is stored in mast cell secretory granules and its activity is potentially controlled shortly after exocytosis by several inhibitors, including α_2M (Raymond et al. 2009; Walter et al. 1999). In turn, ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are peptidases with 19 paralogs in humans, which participate in many functions including processing of pro-collagen, remodelling of extracellular matrix, and organization of connective tissue, coagulation and inflammation (Kelwick et al. 2015). ADAMTSs are controlled by α_2Ms; uncontrolled activity results in pathologies including arthritis and cancer (Luan et al. 2008; Tortorella et al. 2004). α_2Ms also play an important role in hemostasis, by controlling peptidases that participate in blood-coagulation and fibrinolysis. These include factor VIIa, thrombin and plasmin (Petersen et al. 2009; Kremers et al. 2013; Schaller and Gerber 2011). α_2Ms can also function as master inhibitors of cartilage-degrading factors (Wang et al. 2014), and as inhibitors of both the esterolytic and kinin-producing activities of plasma kallikrein (Harpel 1970).

α_2M is also involved in attenuating infection by pathogens and parasite growth in various species by counteracting their peptidases. Secreted peptidases must be neutralized to restrict the steps of invasion, nutrition of parasites, and parasite-mediated inactivation of elements of host immunity (Armstrong 2010). α_2M inhibits several peptidases from bacteria, such as neutral peptidase from *Fusiformis nodosus* (causing ovine foot-root) (Merritt et al. 1971; Werb et al. 1973), collagenase from *Clostridium histolyticus* (causing gangrene) (Giroux and Vargaftig 1978), alkaline peptidases from *Bacillus subtilis* (Dolovich and Wicher 1971), peptidases from *Pseudomonas aeruginosa* and *Serratia marcescens* (causing agents of corneal lesion ulceration and septic shock) (Horvat et al. 1989; Molla et al. 1987; Khan et al. 1995), immune inhibitor A metallopeptidase from *Bacillus anthracis* (causing agent of anthrax) (Arolas et al. 2016), several gingipains from *Porphyromonas gingivalis* involved in periodontitis and arthritis (Gron et al. 1997), the enterotoxin fragilysin from *Bacteroides fragilis* (agent of diarrhoea) (Moncrief et al. 1995), and many other peptidases from important parasites (Borth 1992; Scharfstein 2006). It also protects from injected peptidases, such as the hemorrhagic and collagenolytic snake-venom metallopeptidase from *Bothrops jararaca* and *Crotalus atrox* venom (Anai et al. 1998; Werb et al. 1973). Indirectly, it may also function by delivering foreign proteins to macrophages and enhancing the ability of mammals to produce anti-peptidase antibodies, for example, against the major cysteine peptidase of *Trypanosoma cruzi* (agent of Chagas disease) (Morrot et al. 1997).

The role of α_2Ms in bacteria is still largely unknown. They are found in both pathogenically invasive and saprophytically colonizing species, which suggests that they are involved in colonization rather than virulence (Budd et al. 2004). *In vitro* studies with *E. coli* do not discriminate between ECAM knockout mutants and wild-type strains. Only after exposure to peptidases at physiological concentrations, a decrease in the mutant survivability can be observed. It seems that ECAM protects membrane-attached proteins and other cell components from host peptidases (Garcia-Ferrer et al. 2015). Moreover, in the same operon with ECAM, a penicillin

binding protein (Pbp1C) co-occurs that functions as peptidoglycan transglycosylase for cell wall biosynthesis (Budd et al. 2004). Therefore, these two proteins may be coupled as a periplasmic defence and repair system, acting in a concerted fashion. In addition, in *P. aeruginosa*, *E. coli* and several other strains, a second α_2M homolog (MagD) is present that forms multi-molecular complexes with proteins from co-transcribed genes. Together, they may be involved in bacterial pathogenicity and/or defence against the host immune system, but this remains undefined to date (Robert-Genthon et al. 2013).

6.5 Other Physiological Functions

The role of α_2M is not restricted to peptidase binding and inhibition but is extended to interactions with several other endogenous and exogenous proteins, with important physiological implications (Table 6.2 and references therein). To date, the nature of these interactions were studied biochemically, due to difficulties in the crystallization of α_2M to obtain high-resolution X-ray models. Consequently, the exact interacting surfaces are still unknown. They can be observed in native (e.g. interleukin-6; IL-6) and induced α_2M (e.g. nerve-growth factor-β; NGF-β) after the exposure of cryptic sites by reactions with peptidases (e.g. plasmin or highly reactive amine), and can be located on the surface (e.g. β-amyloid peptide-binding plasma protein; Aβ) or inside the prey chamber (e.g. transforming growth factor-β; TGF-β) (Mettenburg et al. 2002; Webb et al. 1998; Liebl and Koo 1993; Matsuda et al. 1989). So far, hydrophobic, ionic and covalent interactions are reported. TGF-β and other growth factors, cytokines and hormones may form complexes with α_2M *via* a common mechanism involving interactions between exposed tryptophan and/or other hydrophobic residues, and a hydrophobic region in induced α_2M (Webb et al. 1998). Also, after induction, the highly reactive cysteine and glutamine/ate residues of the thioester can form disulphide and/or thioester-based covalent bonds with molecules in close proximity. The presence of the molecules inside the α_2M chamber during induction may be required for trapping, as in the case of insulin that is only crosslinked by the thioester when it concurs with trypsin or elastase (Chu et al. 1991). However, disulphide-based interactions can also be formed at a later stage, after induction, if the molecules are small enough to pass through the narrowed entrances of the induced α_2M, and to reach free cysteines arising from thioester hydrolysis. In many cases, molecules from the same or even different class compete for the same binding site on α_2M, which interferes with complex formation. For example, neurotrophins can be displaced by NGF-β, and heparin interacts directly with TGF-β, thus liberating it from the complex with α_2M and restoring its activity (McCaffrey et al. 1989; Wolf and Gonias 1994). All these binding partners probably share a common mechanism that is dependent on local topological structures rather than amino acid sequences, since most of these ligands lack sequence homology. Besides the amino acid sequence, posttranslational modifications such as glycosylation participate in direct interactions with lectin-like binding proteins in both native and induced α_2M (Arnold et al. 2006).

Table 6.2 α_2M interactions with other molecules and implications

Type	Name	α_2M form/Interaction	Implications of the complex	References
Interleukin and Cytokines	IL-1-β	Induced. Disulphide-based. Promoted by Zn^{++}.	Biological activity is retained, and α_2M may act as a spatial and temporal regulator of IL-1β.	Borth et al. (1990) and Lindroos et al. (1995)
	IL-6	Native. No competence from IL-l-α, IL-10 and IL-2.	Biological activity is retained, and α_2M may act as a carrier protein in the circulation. Protection against peptidases at inflammatory sites.	Matsuda et al. (1989)
	IL-8	Induced. Non-covalent.	α_2M does not inhibit IL-8-induced neutrophil degranulation or chemotaxis and protects it from peptidase degradation.	Kurdowska et al. (1997)
	Leptin	Induced. Hydrophobic-based.	Binding of leptin to induced α_2M and its rapid clearance by the α_2M receptor may significantly influence the bioavailability of leptin in human plasma.	Birkenmeier et al. (1998)
	Tumour necrosis factor-α (TNF-α)	Induced by plasmin or methylamine. Non-covalent.	TNF-α, preincubated with either α_2M-plasmin or α_2M-methylamine, remained a potent necrogen for cultured L929 cells. The complex can be removed from the circulation by the α_2M-receptor pathway.	Wollenberg et al. (1991)
Hormones	Hepcidin	Both	The α_2M-hepcidin complex decreased ferroportin expression in J774 cells more effectively than hepcidin alone.	Peslova et al. (2009)
	Inhibin, activin, follistatin	Mainly induced, except activin that binds efficiently in native form. Disulphide-based.	All the complexes may be removed from the circulation by the α_2M-receptor pathway, but activin can be maintained in the circulation by binding native α_2M.	Phillips et al. (1997) and Niemuller et al. (1995)
	Insulin	Induced. Thioester-based. Only if insulin is present during peptidase induction.	Implications in the regulation of carbohydrate and fat metabolism.	Chu et al. (1991)

Growth factors	Basic fibroblast growth factor (βFGF)	Induced. Disulphide-based. αFGF and TGF-β compete for binding to α_2M, whereas PDGF does not.	The complex does not bind to low affinity FGF binding sites and binds poorly to high affinity βFGF binding sites on BHK-21 cells. In addition, it has decreased ability to stimulate plasminogen activator production in bovine capillary epithelial cells.	Dennis et al. (1989)
	Growth hormone (GH)	Induced. Non-covalent.	GH plays an important role in the control of growth.	Kratzsch et al. (1995)
	Transforming growth factor (TGF-$\beta_{1,2}$)	Mainly induced, except TGF-β_2 that binds both. Interactions between topologically exposed tryptophans and/or other hydrophobic residues and a hydrophobic region in α_2M.	Complexes neutralize the activity in fetal bovine heart endothelial cell proliferation assays and can be cleared from the circulation. The complex can be dissociated in the presence of heparin and recover TGF-β activity.	Feige et al. (1996), LaMarre et al. (1991a), Liu et al. (2001), Webb et al. (1998), and McCaffrey et al. (1989)
	Neurotrophins (NT-3, NT-4), nerve growth factor-β (NGF-β), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF)	Mainly induced. Non-covalent. NGF-β exhibits the strongest interaction.	Protection of NGF-β from proteolysis. Binding of α_2M to NGF-β and its precursor pro-NGF causes neurodegeneration in a p75(NTR)- and pro-NGF-dependent manner.	Wolf and Gonias (1994), Liebl and Koo (1993), Barcelona and Saragovi (2015), and Ronne et al. (1979)
	Vascular endothelial growth factor (VEGF)	In the interior of induced and the exterior of native α_2M. Binds in a different site than TGF-β.	α_2M does not impact the ability of VEGF to induce cell proliferation or upregulate Ca^{++}.	Bhattacharjee et al. (2000) and Soker et al. (1993)
	Epidermal growth factor (EGF)	Induced. Thioester-based.	~	Gettins and Crews (1993)
	Platelet-derived growth factors (PDGF)	Both. Disulphide-based. All the isoforms bind to the same position.	The reversible nature of the PDGF/α_2M complex could allow for targeted PDGF release near mesenchymal cells that possess PDGF receptors.	Bonner et al. (1991)
	Insulin-like growth factor biding proteins (IGFBP1/2)	~	The decreased concentration of the complexes in the circulation provides a greater amount of free IGFBP-2.	Sunderic et al. (2015)

(continued)

Table 6.2 (continued)

Type	Name	α_2M form/Interaction	Implications of the complex	References
Other	Carboxypeptidase B (CPB)	Native. Non-covalent.	Plasma CPB activity is not significantly affected by binding to α_2M. The complex may function as a "shuttle" to modulate the clearance of CPB from circulation.	Valnickova et al. (1996)
	Mannan binding lectin (MBL)	Both. Direct binding of oligomannose glycans of α_2M to the lectin domains of MBL.	The lectin interaction may represent a mechanism for the localization of α_2M to the MBL/MBL-associated serine proteases complex for the inhibition of spontaneously or inappropriately activated serine peptidases in the serum.	Arnold et al. (2006)
	Apolipoprotein E (ApoE)	Mainly induced. Non-covalent. Fats, sphingomyelin, very low-density lipoprotein, PDGF and Aβ inhibit complex formation.	ApoE plays a central role in the transport of lipids among different organs and cell types.	Krimbou et al. (1998)
	Eosinophil cationic protein (ECP)	Induced. Non-covalent.	The interaction with α_2M may reflect a mechanism by which the organism protects itself against the deleterious effects of the highly cytotoxic protein ECP.	Petersen and Venge (1987) and Bystrom et al. (2011)
	Tartarate-resistant acid phosphatase (TRAP)	Both. Ionic interactions	Complex affects both enzyme activity and immunoreactivity. α_2M may modulate clearance of TRAP from the circulation and possibly from areas of inflammation.	Brehme et al. (1999)
	β_2-Microglobulin	Native	Putative participation of α_2M in modulating β_2-microlgobulin metabolism.	Gouin-Charnet et al. (2000)
	Defensin HNP-1	Induced. Disulphide-based.	α_2M may function as a scavenger of defensins and other peptide mediators in inflamed tissues, and may act as a mechanism for the regulation of inflammation.	Panyutich and Ganz (1991)
	Myelin basic protein (MBP)	Both	Complex protects myelin from proteolysis and may protect extracellular compartments from immunogenic myelin fragments. α_2M mediates clearance form circulation.	Gunnarsson and Jensen (1998)
	β-amyloid peptide-binding plasma protein (Aβ)	Induced. Binding to amino acid sequence (1314–1365) corresponding to α_2M receptor domain.	α_2M and its receptor, low density lipoprotein receptor-related protein, function together to facilitate the cellular uptake and degradation of Aβ.	Mettenburg et al. (2002) and Du et al. (1997)

	Osteogenic growth peptide (OGP)	Both	Native α_2M enhances the immediate availability of OGP to its target cells. Induced α_2M may participate in the removal of OGP from the system.	Gavish et al. (1997)
	Lipoprotein lipase (LPL)	Native. Non-covalent.	~	Vilella et al. (1994)
	CpG oligodeoxy-nucleotides	Induced	Complex enhances the immunostimulatory properties significantly, and protects it from degradation by nucleases.	Anderson et al. (2008)
	Chaperonine activity	Both forms and hypochloride-induced.	α_2M is a specialized chaperone that prevents the extracellular accumulation of mis-folded and pathogenic proteins, particularly during innate immune activity.	Wyatt et al. (2013), (2014)
	Interacts with unfolded proteins			
	Limulin	Induced	α_2M inhibits limulin, a sialic acid-binding lectin that is the mediator of hemolysis in the plasma of the horseshoe crab *Limulus polyphemus*.	Swarnakar et al. (2000)
	Protein G related α_2M binding (GRAB)	Native	It is a virulence factor that traps α_2M and regulates proteolytic activity at the bacterial surface and the host-microbe relation during *S. pyogenes* infections.	Rasmussen et al. (1999)
	Protein G	Native	Protein involved in binding of Group G streptococcal strains with α_2M. It is a virulence factor.	Müller and Rantamäki (1995)
	Ricin A-chain	Induced. Disulphide-based.	Ricin is a highly toxic, naturally occurring lectin produced in the seeds of the oil plant, *Ricinus communis*. Complex formation with α_2M decreases the activity of ricin.	Ghetie et al. (1991)
	α_2M from *Pseudomonas aeruginosa* (MagD)	Native	It forms a complex with three other molecular partners encoded by the same operon, MagA, MagB and MagF. It may play a role in virulence and/or bacterial defence.	Robert-Genthon et al. (2013)

By regulating the distribution and activity of many cytokines, hormones, growth factors and other proteins, α_2M modulates cell proliferation and cell survival pathways. In complex, molecules may retain or lose their function, and α_2M may acquire new characteristics with beneficial (e.g. more effective hepcidin or inhibition of eosinophil cationic protein) or detrimental (e.g. inhibition of NGF-β) implications for physiology (Peslova et al. 2009; Barcelona and Saragovi 2015; Bystrom et al. 2011). Non-covalent bound cytokines can be displaced by heparin or specific cytokine receptors and released during tissue injury and inflammation (LaMarre et al. 1991b). Moreover, induced α_2M serves as a system for balancing or preventing the harmful systemic or local effects of excess cytokines, such as TGF-β and tumour necrosis factor-α (TNF-α), and of toxins such as ricin-A, by removing them from the circulation in a concerted system with LRP1 (LaMarre et al. 1991a; Ghetie et al. 1991). In many cases, α_2M protects the bound molecule from lysis by peptidases or nucleases due to steric hindrances (Anderson et al. 2008; Matsuda et al. 1989). Also, it may serve as a chaperone, binding misfolded potential pathogenic proteins and preventing their extracellular accumulation during stress conditions, and particularly during innate immune system activity (Wyatt et al. 2013, 2014). α_2M influences protein function not only through complex formation, but also by direct modifications through enzymatic reactions. Uniquely, human α_2M exhibits esterase activity that acts specifically on ghrelin, a major hormone secreted from endocrine cells in the stomach that is implicated in feelings of satiety and in the body's reward system (Eubanks et al. 2011).

Besides its physiological role in the human body, α_2M is exploited by bacteria for their own wealth. Through two mechanisms, involving protein G and protein G-related α_2M-binding protein (GRAB), bacteria bind α_2M on their membrane surface, thereby protecting important virulence determinants from host-attacking peptidases and proteolytic degradation. This regulation of proteolytic activity on the bacterial surface can affect the host-microbe relation during *Streptomyces pyogenes* infections (Rasmussen et al. 1999; Müller and Rantamäki 1995). Early observations suggest that α_2Ms of bacterial origin interact with several other proteins that are potentially involved in virulence or defence, however, the exact function is still unknown (Robert-Genthon et al. 2013; Budd et al. 2004).

6.6 Conclusions

α_2Ms represent an evolutionary conserved arm of the innate immune system that regulates the distribution and activity of many proteins, including peptidases, cytokines, hormones and many other physiological effectors (Armstrong and Quigley 1999). It is a sophisticated means to spatially and temporally restrict and regulate key physiological processes. Due to its importance in physiology, several efforts were made to understand the mechanism of action *in vivo* and *in vitro* at biochemical and structural levels. However, further information is required to obtain a detailed picture of how it interacts with proteins. Clear knowledge of the molecule

and its interactions at molecular level may allow the rational design of adjuvants, and may also lead to the development of new therapeutic agents against infectious diseases.

Through simple sequence similarity searches, thousands of putative sequences can be identified from both α_2M-type inhibitors and complement proteins. The massive information available, however, still needs to be filtered and verified and more detailed studies at protein level must to be completed to reveal the real extent and functional role of α_2Ms in several organisms. It would not be surprising to identify roles and activities other than peptidase inhibition that involve participation in more complex systems and networks for either housekeeping or protective functions against external attacks. In addition to mammalian homologs, the more recently identified bacterial α_2Ms seem to interact with many other proteins forming macromolecular complexes, which are released after proteolytic attack (Robert-Genthon et al. 2013; Budd et al. 2004). They could serve, therefore, as sensors and signal transmitters for the presence of potentially damaging endogenous or exogenous peptidases, leading to the initiation or pausing of important functions. This could be beneficial to the organism as a fast response to environment changes during parasitism or mutualism.

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