GENERAL INTRODUCTION

THE IMPORTANCE OF PROTEINS IN THE STRUCTURE AND ENERGETICS OF THE CELL

The importance of proteins in living organisms embraces two aspects: structural and functional. From a structural viewpoint, the role of proteins in cellular morphogenesis is fundamental. Indeed, morphogenesis is a process of self-organisation involving self-assembly mechanisms whereby organelles, organs and even whole organisms develop in time and space as a function of genetic information. Such a process is only possible because **one-dimensional information encoded by DNA is translated into three-dimensional information in proteins**. In general, proteins fold spontaneously in their biological environment. In order for cytosolic proteins to attain an active structure, water plays a definitive thermodynamic role. As for membrane proteins, their structure depends on a multiphasic environment. The hydrophobic effect is the driving force in the formation of active protein structure. The stability of protein molecules is maintained principally by hydrogen bonding and hydrophobic interactions, which play a crucial role in the formation of the tertiary structure of soluble proteins and oligomeric structures. An additional stabilising effect is contributed by the association of subunits. This largely involves hydrophobic interactions, but also salt bridges, which can form between subunits and are stabilised by an apolar environment (see GHÉLIS & YON, *Protein Folding*). A higher level of complexity is encountered in multienzyme complexes, in which non-identical subunits bearing different enzyme activities may associate. One significant example of this type of organisation is given by fatty acid synthase, a complex of 7 associated enzymes, in which several levels of structural organisation occur. In yeast, this complex is composed of 2 polypeptide chains and has a molecular weight of 2 300 kiloDaltons (kDa). Chain A (185 kDa) possesses 3 of its enzyme activities: acyl carrier protein (ACP), β-ketoacetyl reductase and the condensing enzyme. Chain B (175 kDa) contains the four remaining enzyme activities: acetyl transacylase, malonyl transacylase, β-hydroxy-acetyl dehydratase and enoyl reductase. Macromolecular assemblies can be even more elaborate heterocomplexes comprising proteins, membranes, ribosomes and viruses.

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Proteins have very varied molecular masses and, therefore, sizes. Table 1 lists examples of the dimensions of some molecules and cellular components. We might ask whether, during the morphogenesis of higher order structures such as organelles and mitochondria, the same self-assembly processes take place. According to certain authors, in particular LEHNINGER, self-assembly alone cannot fully explain morphogenesis; certain pre-formed elements acting as scaffolds must be present. **Therefore, proteins are the basis of all cellular organisation since they translate one-dimensional information into a three-dimensional structure either in an aqueous environment or in the multiphasic environment of the biological membrane.**

Table 1 Approximate dimensions and weights of selected biomolecules and cellular components *(from LEHNINGER, 1972)*

	Dimension (Å)	*Weight (Daltons)*
Alanine	5	89
Glucose	7	180
Phospholipid	35	750
Myoglobin	36	16 900
Haemoglobin	68	65 000
Myosin	1 600	470 000
Glutamate dehydrogenase	130	1 000 000
E. coli ribosome	180	2 800 000
E. coli bacteriophage X174	250	6 200 000
Tobacco mosaic virus	3 000	40 000 000
Liver mitochondrion	15 000	1×10^{-12} grams
E. coli cell	20 000	2×10^{-12} grams
Spinach chloroplast	80 000	1.3×10^{-10} grams
Liver cell	200 000	2×10^{-9} grams

A great number of proteins exist, many of which are enzymes. A cell such as *E. coli* contains around 3 000 different proteins and 1 000 distinct nucleic acids. Plants contain even greater numbers of each and higher organisms yet more. In humans there are about 5×10^6 different proteins. The total number of proteins present in all living species can be estimated to be roughly 10^{11}. We thus find ourselves in the presence of a huge diversity of molecular species. This diversity can be simplified in two ways: by either functional or structural analogy. Discarding all analogous proteins that have the same function in different species and organisms, for example the diverse range of cytochromes c, the number then becomes approximately 10^5. If we ignore functional differences and consider only structurally analogous proteins, the number diminishes to a few hundred. Both of these methods of reduction, that is, by functional or structural analogy, correspond to two different aspects of evolution. The former corresponds to the evolution of species; the latter to the evolution of proteins, or in other words, differentiation.

In terms of function, proteins can support a range of activities depending on their nature and their degree of complexity. Thus, the basic function of all monomeric or oligomeric proteins is **the specific or selective binding of one or more ligands**. This mutual recognition involves a non-covalent interaction between the protein and its ligand, which may be a small molecule or another macromolecule. These interactions play a very important role in many biological processes, including sensory perception. Apart from this primary role, proteins fulfil many functions, for example:

- catalysis, in the case of enzymes,
- motility, exemplified by contractile proteins,
- transport, as is the case for haemoglobin and membrane transporters,
- photoreception, in the case of proteins involved in vision (rhodopsin).

Among these, we are interested principally in functional proteins and more specifically, enzymes, which form the subject of this work.

HISTORICAL VIEW OF THE DEVELOPMENT OF ENZYMOLOGY

The origin of Enzymology coincides with the origin of Biochemistry. In actual fact, biochemistry as a discipline really crystallised around enzymology. However, even though both their roots can be traced back to the beginning of the 19th century, biochemistry and enzymology only began to overlap after much development, and for a long while came up against the ideas of the vitalists. In fact, the existence of enzymatic activity had been known for nearly two centuries. But only at the beginning of the 20th century, after several attempts, was a quantitative theory of enzyme catalysis successfully developed; the theory was first established for a particular case: the mechanism of action of invertin (now called invertase). The law governing the rate of enzymatic reactions developed by Victor HENRI, then MICHAELIS and MENTEN, still remains valid today in its phenomenological form. Later developments represented increasingly wider generalisations of their initial hypothesis. In fact, these first kinetic and thermodynamic approaches remained purely phenomenological for a long time. Structural studies, which have enabled understanding of the relationship between structure and function, were only developed very much later. Nowadays, although enzyme catalysis may be considered as a particular case of chemical catalysis, we should remind ourselves that at one time, when it was not known how to obtain a purified enzyme, many scientists treated enzymatic activity as a manifestation of the vital force.

The first observations were carried out on vegetable and animal cell-free extracts. Thus, in the 18th century SPALLANZANI (1783) discovered the action of gastric juices on the liquefaction of meat. A little later, PLANCHE (1810–1820) showed that a root extract caused the dye gaiacol to turn blue in colour and named the agent responsible for this action cyanogen, although GAY-LUSSAC had already described

C_2N_2. In 1830, ROBINET and BOUTRON-CHALARD succeeded in the hydrolysis of amygdalin using an extract of bitter almonds, which, they suggested, contained an active principle. LIEBIG and WÖLHER (1837), and later ROBIQUET (1838), called this principle emulsin. A certain number of enzymes, termed "ferments" or "diastases", were then identified: salivary diastase, or ptyalin (LEUCHS, 1831), the diastase from malt, or maltase (PAYEN & PERSOZ, 1833), sinigrase (FAURÉ, 1835) and pepsin (SCHWANN, 1836). In parallel to the discovery of these diverse enzymes, the synthesis of urea by WÖLHER in 1828 showed that biochemistry was a branch of organic chemistry. In spite of this, following on from positivists certain authors, including LITTRÉ, maintained that there was no chemistry of the living but only of dead, organic substances. Thus, in the second half of the 19th century, the separation arose between chemistry and biology. Dead, organic substances belonged to the former; to the latter: living, organic substances.

With the work of Claude BERNARD, it became more and more clear that catalysts played a role in metabolic processes. In 1848, BERNARD discovered the role of pancreatic juices and described the action of an albuminous substance, pancreatin. Similarly, in the breakdown of glycogen into glucose, he invoked the activity of a diastase. Claude BERNARD accepted therefore the existence of "soluble ferments", most likely proteins catalysing breakdown reactions. Thus, he wrote in 1878 in *Leçons sur les phénomènes de la vie communs aux animaux et aux végétaux* (*Lessons on the phenomena of life common to animals and plants*): "The generality of diastasic action makes for the archetypal chemical process of living beings *par excellence*." However, he did not explain the syntheses. During the same period some new enzymes were identified and purification trials were attempted. In 1862, DANILEWSKI succeeded in separating trypsin from pancreatic amylase by adsorption of the latter to collodion. BÉCHAMP, in 1864, considered that the transformation of saccharose into glucose involved an enzyme, zymase. Not long after (1886), Raphaël DUBOIS discovered luciferase, the enzyme responsible for bioluminescence. Later, different authors, in particular Gabriel BERTRAND (1896), discovered several oxidative enzymes; G. BERTRAND isolated laccase and showed its specificity for polyphenols having their hydroxyl group in ortho or in para, but not in meta. Laccase is a metalloprotein that contains Mn^{++}, which led BERTRAND to introduce the concept of cofactor. Thus, from this period on, several enzymes started to be identified.

The diverse attempts to explain enzymatic activity could be grouped into two contrasting views. In the first, enzymatic activity was reduced to a simple chemical action, a hypothesis already put forward by BERZELIUS, who wanted to include it in ordinary chemical processes. In the second, enzymatic activity remained a property of living matter, a manifestation of the "vital force". The controversy between PASTEUR and LIEBIG, which sparked in 1870, illustrates these two attitudes. LIEBIG represented the purely chemical theory of enzymatic processes, whereas PASTEUR recognised and thought to have demonstrated that yeast must be living in order to ensure alcoholic fermentation (1871). PASTEUR established, therefore, a distinction

between the non-organised "ferment" like rennin or diastase, and an organised "ferment" like yeast and bacteria, which can lead to lactic acid production. He considered cellular structures indispensable for this action, which thus gave rise to the distinction between an essentially organised "ferment" and a non-organised enzyme. The term *enzyme*, from the Greek *zume* (zyme) meaning "leaven", was introduced by KÜHNE in 1878; enzymes are the principle found in leaven (*en zume*). The PASTEUR-LIEBIG controversy ended in 1897, when BÜCHNER showed that a yeast extract completely lacking cells could just as well stimulate alcoholic fermentation. All the facts provided by PASTEUR were correct, though incorrectly interpreted, and LIEBIG's hypothesis proved to be right. In the same era, in 1898, HILL discovered that maltase could catalyse a reversible reaction, i.e. **an enzyme catalyses equally well a reaction directed towards synthesis as towards degradation**.

In the second half of the 19th century tests to quantify enzymatic activity coincided with the emergence of physical chemistry and above all with chemical kinetics and and thermodynamics, with GUDBERG and WAAGE, VAN T'HOFF and ARRHENIUS. The study of reaction rates formed the rational basis of chemical kinetics and progressively of enzyme kinetics. The path of thought traced by BERZELIUS led O'SULLIVAN and TOMPSON (1880) to study the action of yeast saccharase by quantitative methods. This enzyme converts saccharose into glucose and fructose (laevulose), which is manifested by an inversion of the deviation of plane-polarised light. Digestion of saccharose, a dextrorotatory molecule, gives rise to one molecule of glucose and one molecule of fructose, both of which are laevorotatory. Thus, the authors were able to follow quantitatively the progress of the reaction with the help of a polarimeter. To explain this process, they proposed a first-order law in which the rate of appearance of the hydrolysis products is expressed by the relation:

$$dx/dt = K(a - x)$$

where a represents the total concentration of substrate, x the total concentration of the reaction products that appeared as a function of time, and K, a constant. Integrating this equation gives the following:

$$Kt = \ln a/(a - x)$$

which is characteristic of first-order reactions. **This study probably represents the first truly quantitative approach to the kinetics of enzyme reactions.**

◤ In 1898, E. DUCLAUX showed that even though this relation holds true for a predetermined substrate concentration, there is, however, no proportionality between the reaction rate and the substrate concentration. In other words, the constant K varies with the substrate concentration. DUCLAUX noticed that, conversely, for a very short period at the start of the reaction the quantity of inverted sugar was proportional to time. He suggested that the reaction products acted to slow down the reaction according to the equation:

$$dx/dt = K - K_1 a/x$$

so: $$t = \frac{a}{K_1} \ln \frac{K_a}{K_a - K_1 x}$$

The phenomenon of inhibition by the reaction products had previously been pointed out by TAMMANN.

In reality, neither first-order kinetics nor zero-order kinetics with the inhibitory activity of the hydrolysis products sufficed to explain entirely the experimental facts. In particular, as already indicated by TAMMANN, none of the previous expressions took into account the influence of the enzyme concentration on the reaction rate. Therefore, a more suitable schema remained to be found that would be capable of integrating all the known experimental results, i.e.:

- the logarithmic profile of the kinetics,
- the inhibitory activity of the reaction products,
- the influence of enzyme concentration,
- the influence of substrate concentration. ◢

The basis for the theory of enzymatic reactions rests on the temporary formation of an intermediate complex between the enzyme and substrate. This idea was found to be closely related to the concept of enzyme specificity, the origin of which may be attributed to an observation by PASTEUR, who showed that during the fermentation of DL-tartrate only the D isomer was destroyed. But it was in fact the convergence of early enzymology with organic chemistry that led Emil FISCHER to interpret specificity in terms of molecular structure, at a time when only the structure of the substrate could have been known. In 1894, E. FISCHER carried out a series of experiments proving the influence of the substrate's stereochemical configuration on the enzymatic activity. Furthermore, he showed that the stereospecificity even extends to the inhibition by compounds whose structures are analogous to that of the substrate, which was confirmed by ARMSTRONG (1904) and Victor HENRI (1905). FISCHER thus concluded that a temporary association formed between the enzyme and its substrate; the metaphor that he gave was that of a "*key in a lock*", which had a lasting impact on the notion of enzyme catalysis. The intermediate complex breaks down afterwards, regenerating the enzyme in its initial form –an idea previously put forward by WURTZ in 1881.

◤ The forerunners to V. HENRI, such as A. BROWN (1902), followed by H. BROWN and GLENDINNING (1902), diverged from FISCHER's hypothesis. BROWN accepted the formation of an intermediate complex between the enzyme and a part of the substrate – a complex that exists for a short time – but he was unable to come up with a quantitative expression for the reaction kinetics as he did not specify any relative rates for the formation or dissociation of the complex. BROWN and GLENDINNING included the hydrolysis of starch by amylase in the group of catalysed reactions that form an intermediate complex very rapidly and then break down slowly. The global reaction rate is proportional to the concentration of the complex, which gives a rate curve that starts off linear and then becomes logarithmic. But this explanation was still incomplete, since the authors did not take into account the inhibition by the hydrolysis products. ◢

In 1902, Victor HENRI applied to enzyme reactions the law of mass action as used in catalysis in general. He embarked upon a series of investigations into the action of invertin on saccharose and sought to integrate the ensemble of results into a single schema. For HENRI, the reaction took place in two steps. The first was the reversible formation of an enzyme-substrate complex. The concentration of the complex at any moment is given by the law of mass action. In other words, HENRI made the implicit assumption of a quasi-equilibrium. The second step was the irreversible breakdown of the complex to regenerate the enzyme and to give rise to the reaction products. By incorporating the inhibitory effect brought about by the reaction products, the law that governs the rate of saccharose inversion by the action of invertin could thus be written:

$$v = \frac{k_s m(E)(S)}{1 + m(S) + n(P)}$$

In this expression k_s represents the specific rate constant for the breakdown of the enzyme-substrate complex, m and n the association constants, respectively, for the enzyme and substrate, and for the enzyme and inhibitor, such that:

$$m = (ES)/(E)(S) \quad ; \quad n = (EP)/(E)(P)$$

Therefore, m and n represent the respective affinity constants of the enzyme for the substrate and for the reaction products. V. HENRI showed that this relation could be satisfactorily applied to the system studied when using the empirically determined values of m = 30 and n = 10.

The rate equation takes into account the general nature of the reaction and the diverse factors that influence it. It shows a linear relationship between the reaction rate and the enzyme concentration. Furthermore, it is reduced to a zero-order reaction with respect to the substrate when the substrate concentration is high enough. If there is no inhibition by the reaction products, the rate equation simplifies to:

$$v = \frac{k_s m(E)(S)}{1 + m(S)}$$

V. HENRI checked that this law could be applied to other enzymatic reactions, including the action of emulsin on salicilin and the hydrolysis of starch by amylase. In the case of the latter, he referred to the existence of intermediate compounds.

In 1913, MICHAELIS and MENTEN continued the experiments on the action of invertin with the aim to verify HENRI's hypothesis. They formulated two fundamental criticisms of HENRI's experimentation; namely, that he had neither taken into consideration the concentration of H^+ ions, nor the mutarotation of glucose, which appears firstly in its birotatory form before reaching its final form. MICHAELIS and MENTEN therefore worked under optimal pH conditions for the enzyme, as defined by the experiments of SÖRRENSEN, MICHAELIS and DAVIDSON, and in an adequately buffered solution to avoid any change that might be sensitive to pH during

the reaction. Additionally, they eliminated experimentally the inhibition by the reaction products and thus introduced the concept of **initial rate**. Bearing in mind the modifications arising during the experimental process, MICHAELIS and MENTEN were able to verify the theory of V. HENRI far more satisfactorily than he had ever managed to do by himself. The principal interest of the work of MICHAELIS and MENTEN lies, above all, in the development of a graphical method to determine the values of the kinetic parameters for a reaction, in particular, the value of 1/m. This has since been called the MICHAELIS constant. However, it was necessary to wait until the 1930s and even later for linear graphical representations (HANES, 1932; LINEWEAVER-BURK, 1934; EADIE, 1949; HOFSTEE, 1949).

The hypothesis of HENRI and MICHAELIS assumed that a reaction could only take place if it first formed an intermediate complex between one enzyme molecule and one substrate molecule. Additionally, this kinetic treatment assumed that the equilibrium is rapid relative to the chemical breakdown of the complex. A more general approach was later presented by BRIGGS and HALDANE (1925), and then HALDANE (1930), who made no hypotheses as to the relative values of the rate constants and applied the same schema under conditions of steady state. Furthermore, HALDANE applied the treatment of the steady state to enzyme reactions in which he considered all steps to be reversible. These diverse aspects are expanded further in Part II.

All these authors made the simplified assumption that the substrate concentration is much higher than that of the enzyme. This limitation, although legitimate for a great number of reactions in vitro, became subject to criticism from the year 1943. STRAUSS and GOLDSTEIN (1943) and GOLDSTEIN (1944), studying the acetylcholine-choline esterase system inhibited by prostigmin, rigorously re-worked the mathematical treatment by including none of the prior simplifications. They introduced a novel concept: the specific or reduced concentration of the enzyme and substrate, i.e. the concentration divided by the MICHAELIS constant, K_m:

$$s' = s/K_m \quad ; \quad e' = e/K_m$$

and by putting a = (ES)/e, they obtained the fundamental equations:

$$s' = a/(1 - a) + ae' \quad \text{and} \quad v = k_s a$$

This type of approach did not resurface for a long time. It was simply not applicable to the majority of enzyme reactions under in vitro study, in which the enzyme is present in catalytic concentrations. This is why it has been necessary to wait over 20 years for the problem to be addressed again, taking into account the respective concentrations of the enzyme and substrate within the cell. Thus, SRERE (1967), and later SOLS and MARCO (1970), underlined the fact that, during metabolism in vivo, the substrate concentration is often very low compared to that of the enzyme. This problem reveals its importance again today as we begin to tackle quantitively enzyme reactions in the cellular environment. The theoretical advances show the consequences of this situation in the regulation of the enzyme activities under

physiological conditions (LAURENT & KELLERSHOHN, 1984; KELLERSHOHN & LAURENT, 1985). These aspects of cellular enzymology, which are not classical, form the subject of the final part of this book.

Research into enzyme kinetics, which adopted HALDANE's approach for those enzymes possessing Michaelian behaviour, considered more complicated situations and branched in two directions. The first extended the kinetic treatment of those reactions that take place in the presence of several ligands, i.e. a substrate, an effector, activator or inhibitor, or a second or even several other substrates. Diverse formalisms and analyses were reported by ALBERTY (1956), DALZIEL (1957), WONG and HANES (1962), CLELAND (1963) and BLOMFIELD et al. (1963). The second direction concerned the treatment, at steady state, of enzyme reactions involving multiple intermediates (PELLER & ALBERTY, 1959). The general form of the equations derived in these different instances was practically always of the same type as the MICHAELIS-MENTEN equation; however, the experimental parameters have more complex meanings.

Alongside these studies, the application of fast kinetic methods to the study of enzyme reactions progressively developed. The use of flow methods under conditions of pre-steady state was introduced by CHANCE in 1943 during research on catalase and peroxidase, the aim of which was to find direct evidence for the MICHAELIS complex. In fact, the complexes observed did not correspond to the MICHAELIS complex because they appeared later. Today, we know that the life-span of the first enzyme-substrate complex is too short to be detected by flow methods. Thereafter, numerous enzymological works employing flow methods were described. Later in 1963, EIGEN and DE MAEYER developed chemical relaxation methods, which they applied to enzymatic reactions. These techniques, reaching a time-scale three orders of magnitude shorter than with flow methods, made it possible to detect intermediates having a life-span of the order of a microsecond. Ever more precise knowledge of the intermediate steps arising during enzymatic reactions has largely contributed to the conceptual development of enzyme catalysis – and all the more so with the progress in understanding enzyme structure.

While all these phenomenological aspects of studying enzymatic reactions were being advanced, progress in the identification, purification, and determination of the sequence – then later the three-dimensional structures – of proteins has gradually led to the interpretation of enzyme function in terms of structure. First of all, the crystallisation of urease by SUMNER in 1926 marked an important date in the history of enzymology. KUNITZ and NORTHROP followed immediately with the crystallisation of some pancreatic enzymes, which put an end to the controversy sustained by vitalists, as irrefutable proof was provided of the proteinaceous nature of enzymes.

Structural knowledge expanded from the determination of global properties, such as molecular mass, size, form and electrical charge on proteins thanks to the development of hydrodynamic methods and electrophoresis, to which we associate

principally the names SVEDBERG and TISELIUS. Later on, the development of optical methods permitted delving a little more into structural details. The resolution of the amino acid sequence of the insulin molecule by SANGER, from 1954 onwards – the first important date in primary structure determination – marked decisive progress in protein chemistry. Increasingly, the gradual identification of amino acid side chains participating in catalysis enabled a better understanding of how a few enzymes functioned. From this point on, enzymology developed in terms of the structure-function relationship, leading to a progressive evolution in the perception of enzyme reactions. The static image of a "lock and key" was substituted by the notion of molecular flexibility, which enables a protein to adapt to its substrate and thus contributes to the efficiency and selectivity of enzyme catalysis. This idea is illustrated by the "induced-fit" theory introduced by KOSHLAND around the 1950s. Also interpreted in terms of structure are the deviations from MICHAELIS' law observed for certain enzymes, following studies of the cooperative behaviour of haemoglobin in binding oxygen. As in the case of enzyme reactions having Michaelian behaviour, the initial development was purely phenomenological before the introduction of allosteric models by MONOD, WYMAN and CHANGEUX in 1965, and then by KOSHLAND, NÉMÉTHY and FILMER in 1966. These models and their variations are dealt with in Part V.

As a result of crystallographic studies, a new degree of precision was achieved in the interpretation of enzyme activity; descriptions of biological macromolecules were achievable at the atomic scale. It is necessary to cite the remarkable works of L. PAULING around the 1950s, lying at the origin of all these advances, which established rules for the formation of regular structure within biological macromolecules and, more particularly, in polypeptides and proteins. We will never be able to emphasise enough the pioneering role played by PAULING in the progress of all of modern biology. After haemoglobin by PERUTZ (1960) and myoglobin by KENDREW (1960), proteins that certain people considered to be *vestigial enzymes*, lysozyme was the first enzyme whose structure was solved; we owe this result to the group of PHILLIPS at Oxford (1965). Thereafter, numerous crystal structures were determined, and to date the three-dimensional structures of thousands of enzymes are known at atomic resolution. The works of crystallographers allow us to know the spatial positions of all atoms in a molecule as complex as an enzyme. The acquisition of such precise structural knowledge, permitting the visualisation of the topology of enzyme active sites, marked an important step in the understanding of their functional properties, although this has led, albeit over some time, to quite a static representation of protein architecture. The development of high-field Nuclear Magnetic Resonance (NMR) in the 1980s offered a new tool for structural studies that enabled protein structure to be probed in solution. This method has produced a wealth of information for the analysis of the catalytic mechanisms of diverse enzymes.

Protein structures determined by X-ray diffraction in fact represent time-averaged molecular views. In reality, proteins display varied internal motion covering a

time-scale spanning from the nanosecond to the second, or even longer depending on the amplitude of the movement (LINDERSTRØM-LANG, 1955; WEBER, 1975; CARRERI et al., 1975, 1979; COOPER, 1979; YON, 1982). It is clear that, were proteins rigid objects, an oxygen molecule would never penetrate as far as to the iron in haemoglobin and myoglobin, the haem group being buried deeply within the structure (KARPLUS et al., 1979). Nowadays, thanks to the methods of structural refinement, crystallographers are able to evaluate these movements by determination of temperature factors or B-factors. Thus, for proteins like myoglobin (FRAUENFELDER et al., 1979), hen egg-white lysozyme (ARTYMIUK et al., 1979) and the trypsin/trypsinogen system (FELHAMMER et al., 1977; BODE, 1979), it appears that the flexible parts of the molecule, where movements of the largest amplitude take place, are localised to the enzyme active site. This suggests that these movements are of primary importance for the expression of enzymatic activity. With this type of study and molecular dynamics simulations, the fourth dimension, i.e. time, was introduced to protein structure and, in particular, to enzyme catalysis and its regulation.

Aside from these ever more elaborate molecular aspects, the cellular aspects of enzymology are being progressively developed, and can now be addressed at a rigorous level of description. The technological progress made is beginning to permit the application of reasoning methods and molecular enzymological techniques to the study of enzymes either associated, or associating transiently, with cellular structures in the cell. The physico-chemical approach to enzyme behaviour *in situ* corresponds to the original and also very current trend in enzymology. The fact that enzymes are often found inside cells at high concentrations and that they are frequently associated to other enzymes in multifunctional complexes or to cellular structures having a polyanionic character, such as membranes or cell walls, modifies their kinetic behaviour with respect to that measured in solution *in vitro*. All of these problems in cellular enzymology are set to be considerably addressed in the coming years. It is nowadays possible from a rational basis to tackle the differences between the behaviour of enzymes in solution and when linked to cellular structures. Thus, the partisans of the cellular theory from the last century would find, in the near future, more rational justification of the role of the cellular environment in enzyme function, whereas their belief was the product of vitalist perceptions of the era. If some of their assertions contained a hint of truth, the principles on which they were based would be no less incorrect.

Modern enzymology is progressing, therefore, along two main branches. The first is the high-resolution molecular aspect, which includes the temporal dimension of structure. The second is the cellular aspect, which takes into account the cellular medium in which catalytic activity and its regulation occur, in all its complexity.

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