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Effective Kinetic Methods and Tools in Investigating the Mechanism of Action of Specific Hydrolases

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1. Introduction

1.1 The idea of the reaction mechanism

The idea of mechanism of a catalyzed (e.g. enzymatic) and/or uncatalyzed reaction requires understanding and enough experience on the reaction intermediates, their sequence, structures and energetic interactions in drawing all necessary kinetic steps without chemical ambiguity. (Knowles, 1976).

1.2 How essential is the investigation and identification of a reaction mechanism?

The investigation and identification of a reaction mechanism is essential in the sense that it provides information as to the molecular species of the reaction, and it is strongly based on the number and types of the phases involved; however, this discrimination is not always sharp (Vaimakis & Papamichael, 2002). Additionally, the kinetic mechanisms of enzymatic reactions provide evidence of how these biocatalysts could bind with their substrates to accomplish catalysis; thus the molecular machinery of enzyme action can be explained (Papamichael & Theodorou, 2005).

1.3 The importance of an enzymatic hydrolysis mechanism in science and technology

The importance of an enzymatic hydrolysis mechanism in science and technology is now well documented. The third class of enzymes plays an important role in the enzymatic scientific and industrial processes so far, as about 80% of the working enzymes are hydrolases catalyzing the cleavage of C-O, C-N, C-C and some other bonds, including P-O bonds in phosphates (Liese et al., 2000). Numerous hydrolases are now known, and they have been classified into many sequentially and structurally unrelated clans and families. Hydrolases are found in all living organisms, performing functions from simple digestion to regulation of the immune response, blood coagulation and glucose homeostasis (Bachovchin, 2001). Most of hydrolases are used in processing reactions, degrading proteins, carbohydrates and lipids in

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many scientific and industrial disciplines. "How these enzymes work?" is a question that has attracted big attention over past decades (Henrissat, 1991, 1995).

1.4 Enzyme kinetics

The section of biochemistry, which deals with enzymes, is termed "Enzymology", whose a branch is known as "Enzyme kinetics" relevant to the study of enzyme mechanisms, their reaction rates and the conditions which affect these rates. Enzyme kinetics is a particular case of chemical kinetics as enzymes are acting as catalysts, i.e. they increase the rate of a reaction without modifying its overall standard Gibbs-energy change (NC-IUB, 1983). The rate of an enzymatic reaction is expressed as the change in concentration of one of its substrates or products versus time, and it may be the function of several parameters including enzyme and substrate concentrations, time, pH-value and temperature of the reaction medium, as well as of others, affecting the reaction rate. The study of various kinds of models of enzymatic reactions attain of great interest in research, as well as in industrial applications of these biocatalysts, enhancing our knowledge about these processes and/or estimating the values of useful variables and parameters of these systems (Hogan & Woodley, 2000). The rate equation of an enzymatic reaction is a mathematical expression illustrating the catalysis in terms of rate constants and reactant concentrations, and it should best fit the experimental data. Additionally, an enzymatic kinetic mechanism provides evidence of how enzymes and their substrates could be combined in order to accomplish catalysis, as well as it explains the molecular machinery of enzyme action; its knowledge is required in order to be understood how enzymes perform catalysts as well as how their catalytic function could be regulated, and thus to provide information on the nature of the transition states, the geometry of the enzyme's active site, the substrate specificity, the acidic and/or basic groups associated with catalysis, the possible allosteric properties, the mode of regulation, etc.

2. Prerequisite knowledge

2.1 Catalysis by proteases, amylases, lipases, and their generally accepted mechanisms

2.1.1 Catalytic motifs and sequences of two, three, etc, subsites, substrate specificity, example mechanisms and oxyanion hole

Although the catalytic motifs of several specific hydrolases, which are discussed in this context, may be regarded as generally being similar in structure, however a deeper observation reveals that their functional reality is unambiguously completely different, even though similar groups of two, three, etc residues constitute their catalytic sites. Moreover, with respect to catalysis by hydrolases, the term "subsite" was largely brought into general use from proteases (Schechter & Berger, 1967) whose substrates are composed by amino acid residues whose side chains is assumed to interact with specific subsites, i.e. parts of the active site of protease. The conceptual idea of subsite as fundamental mechanistic feature of the enzymatic hydrolysis originated from glucoamylases; quantitative theoretical and experimental kinetic measurements were applied firstly in *Rhizopus* glucoamylases whose binding modes of substrates and their analogues, as well as the subsite interactions were elucidated and important parameters were estimated and reported in a series of inspired

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works (Hiromi, 1983). But, how subsites could function? Dynamic and mechanistic manipulations take place within the active sites due to the reorganization of important residue side chains related to the structures of subsites (Buckle & Fersht, 1994). An enzymatic active site comprises a dual role, i.e. the binding of substrate onto subsite, and the reaction performed by the catalytic residues, while the effectiveness of these roles determines the specificity of enzyme towards its substrate. Let's start this journey with proteases (EC 3.4.x.x), which are distinguished in endopeptidases or proteinases (EC 3.4.11-99.x) and exopeptidases (EC 3.4.21-99.x) (Rawlings & Barret, 1994). The most of endopeptidases are classified into four groups: serine proteases (EC 3.4.21.x), cysteine proteases (EC 3.4.22.x), aspartic proteases (EC 3.4.23.x), and metalloproteases (EC 3.4.24.x) (Rawlings & Barrett., 1993). Furthermore, glycosidases hydrolyze the glycosidic bonds; the enzymes of α-amylase, α-(1→4)-D-glucan-4-glucanohydrolase, family catalyze the hydrolysis and/or trans-glycosylation at the α-1,4- and α-1,6-glucosidic linkages. Furthermore, lipases (triacylglycerol acylhydrolases, EC 3.1.1.x) have not completely defined, as lipolysis takes place at the lipid–water interface of biphasic systems. The example in figure 1 represents the active site of a papain.
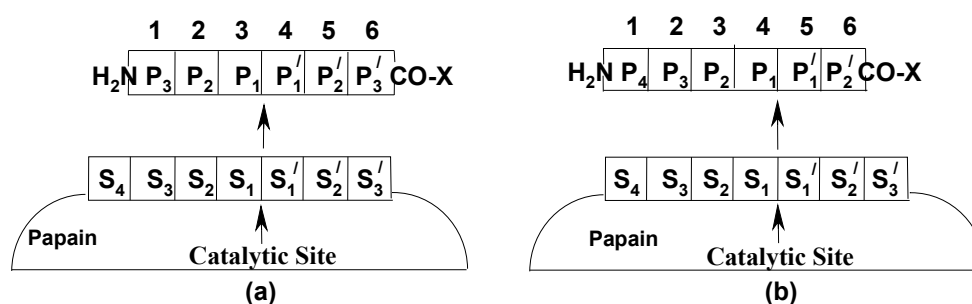

Fig. 1. Illustration of papain catalytic site, which is composed of seven "subsites" i.e. S_1 - S_4 and S_1' - S_3', located on its both sides; positions P, on substrate are numbering similarly as the subsites that they occupy, and are counted from the point of cleavage. When a hexapeptide binds onto papain, then, in **(a)** are formed two tripeptide molecules, while in **(b)** one tetrapeptide and another dipeptide are formed (modified from Schechter &. Berger, 1967).

Additional examples could be referred about the subsites of trypsin where an aspartyl-carboxylate group improves the binding of a lysine side chain. More specific examples of subsites could be found in matrix and other Zn^{2+}-dependent metalloproteinases, whose subsite function is facilitated by three histidines chelating the metal cation; the metal cation behaves as oxyanion hole where a glutamate residue (E^{202} - thermolysin numbering) resembles to a subsite as appeared in figures 2(a) and 3(b) (Auld, 1997; Pelmenschikov & Siegbahn, 2002).

It has been reported that the active site of α-amylases comprises 5–11 subsites, each interacting with one glucose unit of the substrate, and they were designated from **A** to **K**, while the reducing end of the α-glucose chain is located towards **K** subsite (Muralikrishnaa & Nirmala, 2005). However, in amylase-like glycosidases, we should understand a subsite noticeably differently, as these enzymes break more than one glycoside bond without dissociation of the **E-S** complex due to multiple or repetitive attack mechanism, where the enzyme is moving along the polysaccharide chain (sliding), as illustrated in figure 3 (Muralikrishnaa & Nirmala, 2005; Mazur & Nakatani, 1993).
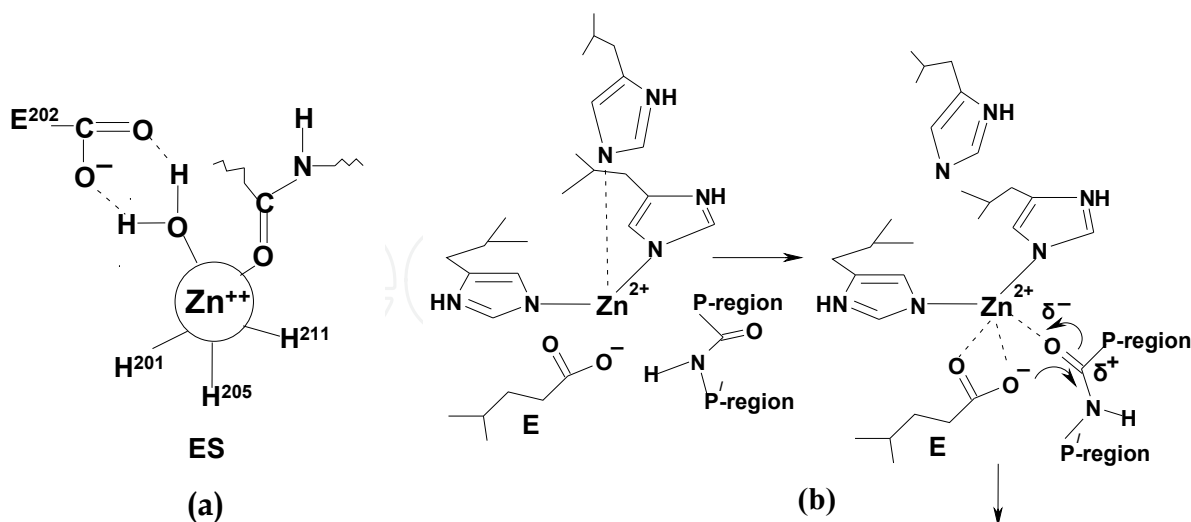
Fig. 2. Catalytic motifs of thermolysin-like metalloproteases: (a) a H_2O chelates Zn^{2+} as part of the catalytic motif, (b) no H_2O participate in the catalytic motif (modified from Auld, 1997, and Pelmenschikov & Siegbahn, 2002, respecively).
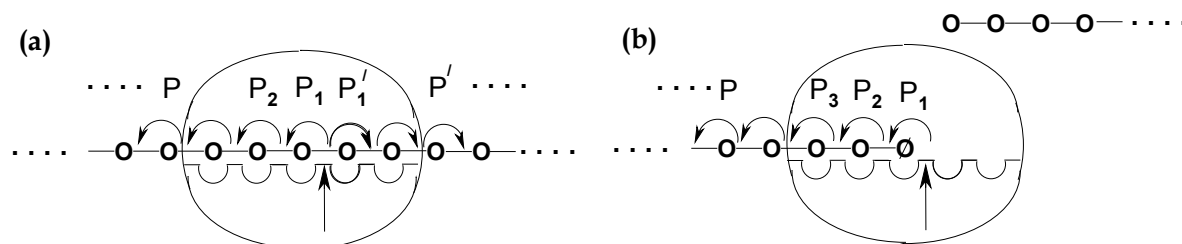

Fig. 3. Complexes of α-amylase with a polysaccharide substrate: (a) is the initial and (b) the same complex after a bond break (**O** and **Ø** are glucose and reduced glucose residues, respectively) (modified from Mazur & Nakatani, 1993).

The catalytic motifs of lipases are similar to those of serine proteases, and are expected analogous effects in the case of subsites. Although this is true, however it should be taken into account that: (a) the natural substrates of lipases are lipids, (b) lipolysis takes place at the water/lipid interface of biphasic (heterogeneous) systems, and (c) lipases should be equipped with the appropriate structural tools as it is the lid domain which plays an essential role in substrate selectivity towards triglycerides, and along with other structural features of lipases undergo a local reorganization to allow free access of substrate onto the subsites. Hence, in heterogeneous reaction media, where a physical adsorption of enzyme on the lipid interface occurs including activation by the lid opening, lipases catalyze reactions by different enzyme-substrate binding modes (Van Tilbeurgh et al., 1993). It should be underlined that the hydrolytic effectiveness of catalytic motifs varies among hydrolases even within the same family. On the other hand, a huge variety of substrates have been synthesized for hydrolases providing complete maps specificity and allowing the

detection of interactions between substrate subsites; casual examples of synthetic substrates for different hydrolases can be found in the literature (Gosalia et al., 2005; Papamichael et al, 1999; Dune et al, 1986; Ohtaki et al, 2001).

An enzymatic reaction proceeds to its accomplishment through several sequential steps comprising the formation of intermediates; this course of action could be designated as a mechanism. The series of sequential step-reactions is likely to be accelerated by the functional groups found in the active site of enzymes. Thus, in enzymatic reactions, the catalysis is moved on due to conformational changes in the enzyme and/or the substrate molecule (Palfey, 2004); during the catalysis, hydrolases change the molecularity of the reaction, which although starts with two reactants (E and S, i.e. bimolecular), however, after the formation of the ES-complex, the reaction continues as unimolecular (Buckle & Fersht, 1994). Fundamental in serine proteases (chymotrypsin-like) is that they maintain one serine (**S**) residue in their catalytic motif which is commonly complemented by two more residues (aspartic acid **D**, and histidine **H**). It is generally accepted that the alcoholic oxygen of **S** plays the role of nucleophile. A functional example could be the triad D^{102}, H^{57}, and S^{195} (chymotrypsin numbering) (Auld, 1997; Bachovchin, 2001). In cases of more specific substrates a negative charge is spread all over the catalytic motif of these enzymes, designated as "Charge Relay System", whose a structural characteristic is the uncharged imidazole aromatic ring of the catalytic **H**. In cases of less specific substrates, the negative charge is localized on the alcoholic oxygen of S^{195} (the nucleophile) while the imidazole ring of catalytic **H** remains positively charged (general acid-base catalysis) (Hunkapiller et al.,1976); in both cases the development of an "oxyanion hole" is a prerequisite for catalysis by serine proteases. At a first glance, cysteine proteases perform catalysis similarly as serine proteases do, while one cysteine (**C**) residue is fundamental in their catalytic motif; regularly, cysteine (**C**) residue forms an ion-pair along with a histidine (**H**) residue (C^{25}-S^- /H^{159}-Im^+H - Papain numbering ion-pair) (Rawlings & Barret, 1993,1994). In cysteine proteases, acylation proceeds through the formation of an anionic tetrahedral adduct, the enzyme-substrate (**ES**) complex, while an oxyanion hole may be developed although it is not a prerequisite for catalysis by these enzymes (Theodorou et al., 2001). However, cysteine proteases do not perform catalysis via a catalytic dyad but through more complicated ways whose the main feature is the development of a hydrogen bond (Theodorou et al, 2007a). These latter are shown in figure 4.

Aspartic proteases (EC 3.4.23.x) have a long and perplexed history in Enzymology; the most of aspartic proteases perform catalysis by means of two eponymous aspartyl residues, which are found in opposite states of protonation. Both catalytic aspartyl residues are located in deep clefts formed at the interface of two lobes (e.g. porcine pepsin) (Polgár, 1989). It has been reported that a water molecule attacks the carbonyl carbon of the scissile bond, serving as a third catalytic component along with the active aspartic carboxyl groups, in the catalysis by aspartic proteases (Rebholz & Northrop, 1991); two catalytically active aspartate residues are in either a right or wrong protonic state, involving general base-catalyzed attack by a water molecule on the carbonyl carbon of the scissile bond. Recent ab-initio molecular dynamics simulations on HIV-1 protease were focused on the catalytic D^{25} and $D^{25\prime}$, and resolved all the uncertainties within a unifying hypothesis, as illustrated in figure 5 (Piana & Carloni, 2000).
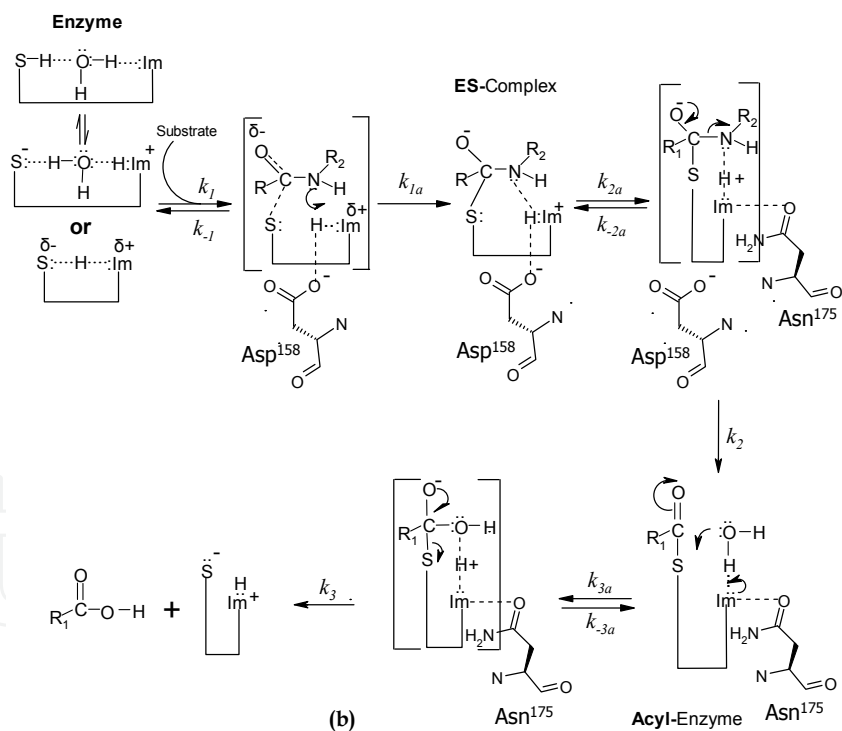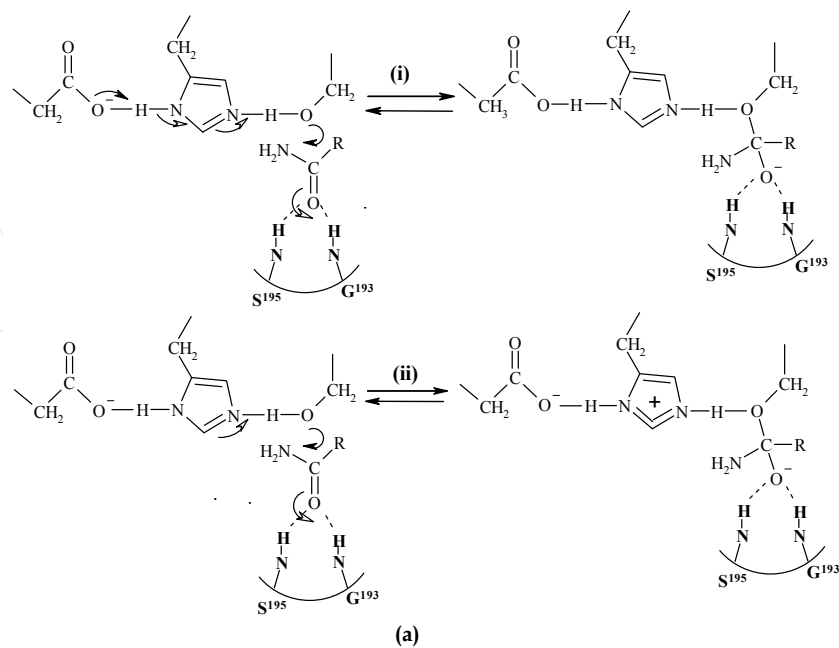
Fig. 4. **(a)** dual function of catalytic triad in serine proteases, where catalysis is performed: (i) by the charge relay system, and (ii) by general acid-general base (the oxyanion hole is formed by **G193** and **S195** residues), **(b)** function and role of the catalytic residues **C25**, **H159**, **D158** and/or **N175** (in bromelain) in cysteine proteases of papain family; a hydrogen bond necessary for efficient catalysis is developed between $D^{158}_{COO^-}$ and/or $N^{157}_{O^{\delta 1}}$, and the positively charged **H159**.

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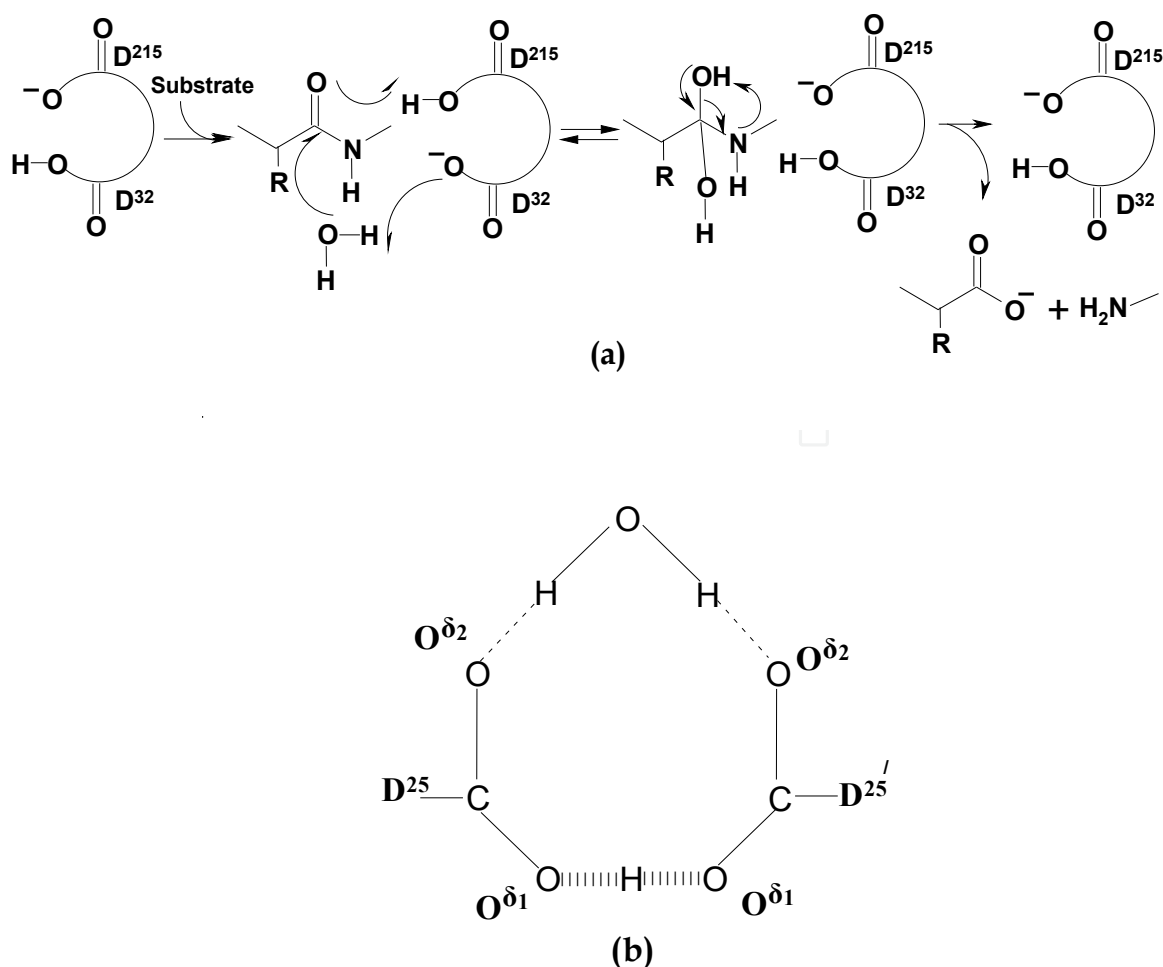Investigating the Mechanism of Action of Specific Hydrolases
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(a)

(b)

Fig. 5. (a) illustration of the catalysis by pepsin, where D^{32} was protonated before first turnover, while one proton is transferred from D^{215} to D^{32}; (b) the catalytic aspartates of HIV-1 protease in a coplanar arrangement where a LBHB joins the $O^{\delta 1}$ oxygens and the H_2O and the $O^{\delta 2}$ oxygens are normally hydrogen bonded (modified from Northrop, 2001).

A variety of methods were applied in studying zinc or other matrix thermolysin-like metalloproteases (**EC 3.4.24.x**), focusing on how protein modulates the properties of zinc cation to achieve specificity and catalytic efficiency. It is well known that bivalent zinc allows many ligands and coordination geometries, and it is unaffected by oxidant or reductive reagents. Nitrogen, oxygen and/or sulfur donors form complexes with the zinc cation; additional ligand to the catalytic zinc cation may be considered one water molecule whose ionization and polarization provides hydroxide ions at neutral pH, while its displacement leads to acid catalysis by the zinc cation (Auld, 1997; Pelmenschikov & Siegbahn, 2002). By means of molecular dynamics, a novel catalytic mechanism has been reported for the metalloproteases, as based on the formation of an anhydride intermediate where a **E**-residue, also, chelates the zinc cation as part of the catalytic motif of these enzymes. Both cases are illustrated in figure 6. The important with metalloproteases is that although two apparently contradicting mechanisms have been suggested, however, both they share common features; the zinc cation is penta-coordinated and it behaves also as the oxyanion hole (Manzettia et al, 2003; Pelmenschikov & Siegbahn, 2002).
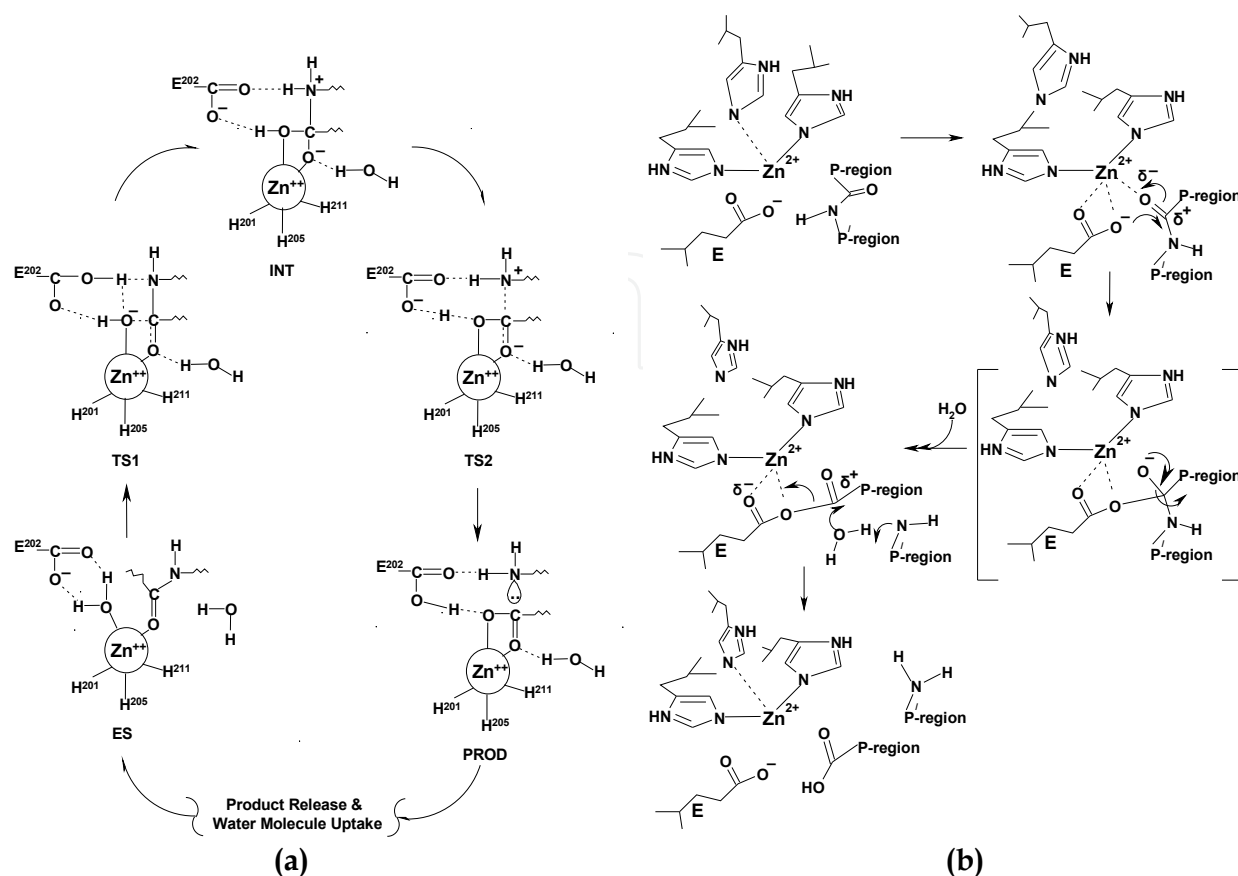
Fig. 6. Matrix metalloproteases: initially, **Zn^{2+}** is three-coordinated and continues as penta-coordinated either (a) with three **H$^{\epsilon 2}$-nitrogens**, the P$_1$'-carbonyl oxygen and one H$_2$O-oxygen or (b) with two **H$^{\epsilon 2}$-nitrogens**, the P$_1$'-carbonyl oxygen and two oxygens of catalytic **E**-residue (modified from Pelmenschikov & Siegbahn, 2002).

Amylase-like glycosidases perform catalysis by hydrolyzing glycosidic bonds either with net inversion of the α-anomeric configuration or with net retention. Glycosidases, either retaining or inverting, catalyze the same overall reaction both by employing acid-base catalysis while the former employ also the covalent catalysis; they are equipped with four highly conserved sequence regions containing all the catalytic residues and most of the substrate binding sites. Although retaining glycosidases perform catalysis by either a glutamate or aspartate, functioning as possible nucleophiles (Svensson, 1994), however, another set of catalytic residues is found in α-amylases, and other related enzymes. It is a triad of carboxylic acids (**D^{197}**, **E^{233}**, **D^{300}** - porcine pancreas α-amylase numbering) (Qian, 1994), where two of them (**E^{233}**, **D^{300}**) are most probably involved in acid catalysis and **D^{197}**, the putative nucleophile providing further electrostatic stabilization of the transition state (Mc Carter &Withers, 1994). In more details, the hydrolysis of glycosidic bonds by glycosidases proceeds by means of a general acid catalysis requiring a proton donor (electrophile) and a nucleophile (the base). In both cases the position of the electrophile is closer to the glycosidic oxygen, and although the nucleophile is closer to the anomeric carbon, in the retaining mechanism, however, it is more distant in the inverting mechanism (Davies & Henrissat, 1995). Oxyanion holes have not been identified in the mechanism of

action in these enzymes, so far. Examples of the retaining and inverting mechanisms are
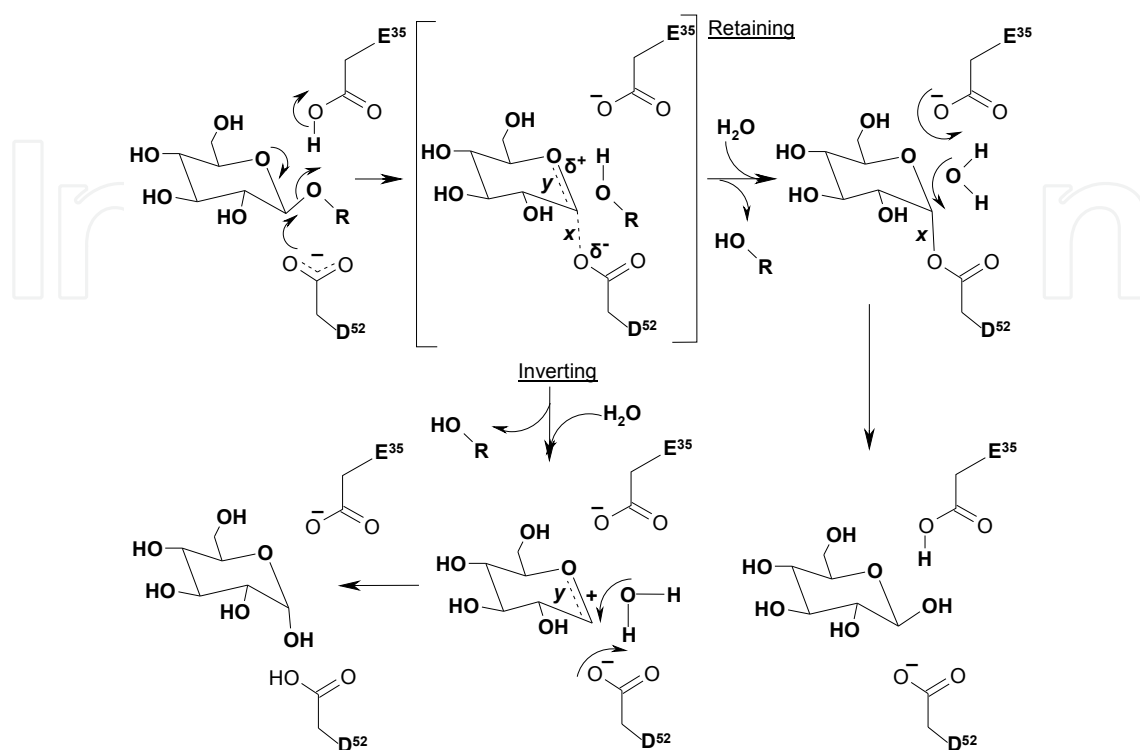shown in figure 7 (Kirby, 2001).

Fig. 7. Retaining and inverting mechanisms of glycosidases: Only one of the dashed bonds (*x*
or *y*) of the intermediate (in brackets) actually occurs and defines the mechanism, i.e. *x*
occurs with the retention mechanism, while *y* occurs with the inversion mechanism
(modified from Kirby, 2001).

Lipases hydrolyze carboxylic ester bonds, and mainly convert tri-glycerides into di-
glycerides or mono-glycerides, and fatty acids and glycerol. Additionally, lipases behave as
esterases catalyzing also esterification, interesterification and transesterification reactions in
nonaqueous media. The reaction course followed by a lipase in its catalytic function
depends on the medium (aqueous or nonaqueous) in which reaction takes place. In media of
low water content or in non-aqueous systems, lipases catalyze synthetic reactions (e.g.
esterification etc) provided that water should be continually removed (Salleh et al, 2006). It
is generally accepted that lipases carry out catalysis via a catalytic triad consisting of the
residues S^{162}, H^{263} and D^{176} (porcine pancreas lipase numbering) (De Caro et al, 1981),
though there are cases where an **E** has replaced the **D** residue. Although, there is little
homology among the known sequences of lipases, however, there are evidences indicating
the convergent nature of the catalytic motifs of serine proteases and lipases (Ollis et al,
1992). Another feature of lipases which has been identified as essential for hydrolysis in
nonaqueous media, is a surface loop, the lid domain, which covers the active site of lipases
(Aloulou et al, 2006). Mechanistic features, similar to those of serine proteases, have been
reported also for lipases, as it is the oxyanion hole, although its development differs in these
enzymes due to their structural particularities (Aloulou et al, 2006). Recently, a full
mechanism of action for the lipase from bovine pancreas (PPL) has been reported and it is
analogous to this reported for serine proteases (figure 4a) (Kokkinou et al., 2011). However,

it is essential to show that the mechanism of fatty acid ester hydrolysis by lipases in micelles, small aggregates or emulsion particles is noticeably different. It should be emphasized that Michaelis-Menten kinetics is applied only in isotropic reaction media, and thus alternative models have been suggested comprising two steps, i.e., a physical adsorption of lipase at the aqueous/lipid interface leads to its activation (opening of the lid) and the formation of the enzyme-substrate complex, and then, the hydrolytic reaction give the products and the adsorbed free enzyme (Aloulou et al, 2006; 1994; Verger & de Haas, 1976). Actually, a two-dimensional Michaelis-Menten catalytic step occurs when the soluble lipase (**E**) is adsorbed on the aqueous/lipid interface (**E***) and binds a substrate molecule resulting the development of the **E*S** complex; then, the soluble product **P*** is immediately diffused in the water layer (**P**). All these are illustrated in figure 8.
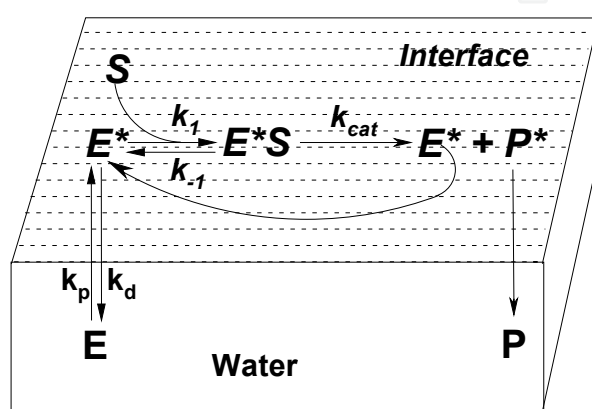

Fig. 8. Illustration of a water soluble lipase catalyzing an interfacial reaction, acting on an insoluble substrate; the asterisk denotes species onto the lipid/water interface, and **E**, **S**, **E*S**, and **P** are the free enzyme, free substrate, enzyme-substrate complex and product, respectively, while **k$_p$** and **k$_d$** are constants associated with the adsorption/desorption of enzyme between aqueous and lipid/water interface (modified from Aloulou et al, 2006).

Overall, the concept of the catalytic motif was not presented as a steady but rather as a dynamic ontologic entity in this context, independently of the number of contributing residues. Thereupon, it seems as more accurate that serine as well as cysteine proteases perform catalysis not by means of the routinely known catalytic triad and or dyad but through extraordinary manipulation of up to five catalytic residues. A similar treatment is worth for aspartic proteases and metalloproteases (Auld, 1997; Rawlings & Barret, 1994; Rebholz & Northrop, 1991; Svensson, 1994) showing that in general, the viewing of an enzymatic mechanism should not be focused only on the markedly referred catalytic residues. The concept of the catalytic sequence gets of special interest, when we have to do with α-amylase-like enzymes as it is illustrated in figures 3(a) and (b), where what extraordinary happens is that each monomer of polymeric substrates acts as catalytic residue! Is not it amazing?

2.1.2 Intermediates and acyl-enzymes

A significant selection of reports has been published, which deals with the mechanisms of action of specific enzymes. The idea of an enzymatic reaction mechanism has been already introduced, herein, where the knowledge of possible intermediates, their sequence, and

structure was underlined. Therefore, understanding enzymatic catalysis it means to predict pathways and rates of enzyme-catalyzed reactions, i.e. to study reaction intermediates and to investigate enzyme structures. Enzymatic reactions which can be described by the Michaelis-Menten model equation they can be also properly divided into steps. For example, in homogeneous reactions at least three main steps may be distinguished, i.e. enzyme-substrate binding, main reaction, and splitting to products and free enzyme; within the latter may occur more than one elementary steps proceeding by means of additional intermediates. Therefore, the term "intermediate" does not comprise a unique notion, and some examples have been already given within this text. An inspection in figures 3 - 7 is enough to show a diversity of **ES** intermediates and structures; anionic intermediates are shown in figures 3(a,b) which are breaking down through transition states, where heavy atom reorganizations and proton transfers occur, and/or covalent intermediates precede the formation of the reaction products in figures 6 and 7. In case of glycosidases (figure 8), the intermediates have the form either of complicated covalent molecular species or of cationic structure (Hiromi, 1983; Ishikawa et al, 2007). By taking into account the concept of the intermediate, it is easier to understand the importance of acyl-enzymes and how these molecular species contribute in the overall catalysis. Nevertheless, an acyl-enzyme is developed, and destroyed (within an enzymatic mechanism) through a nucleophilic attack in most cases, and this is a matter of specific treatment comprising complicated series of extraordinary techniques (Papamichael et al.,2009; Theodorou,2001,2007a).

2.1.3 The low-barrier hydrogen bond (LBHB)

Before closing this topic, an emphasis should be given in the important role and the catalytic contribution of hydrogen bonding within the mechanism of action, in all cases of hydrolytic enzymes, which should provide optimization of the binding energy in the transition state; this latter can be achieved by hydrolases through their functional groups which extend ionic, hydrogen-bond and other interactions. Hydrogen atoms that are bonded to heavy electronegative atoms, being at a short distance from a Lewis-base, may form hydrogen bonds which are mostly ionic in character (Gosalia et al, 2005). Generally, there are certain structural and environmental prerequisites for the development of a hydrogen bond; indicative examples could be (a) the shell of solvent-water, which surrounds and stabilizes the bio-molecules in aqueous solutions, (b) subtle conformational changes due to enzyme-substrate binding interactions, (c) interactions between catalytic residues (e.g. between **C**[25] and **H**[159] in papain-like proteases), which form nucleophilic species, (d) significantly altered pK_a-values of side chains of important residues, and so forth. Nevertheless, the dominant characteristics of a hydrogen bond depend upon the corresponded pK_as of the electronegative atoms sharing the hydrogen and some structural examples could verify the previous sentences (Northrop,2001); a network of hydrogen bonding is contributing significantly in the catalysis either by proteases and lipases, in figure 5, 6(b), 7(a), and 8, including the development of the oxyanion hole, whose hydrogen bonds are stabilized due to a short-lived negative charge on the carbonyl oxygen of the substrate. However, an outstanding type among hydrogen bonds is the low-barrier-hydrogen-bond (LBHB) which has been proved a useful tool in understanding enzymatic catalysis. LBHBs in transition-states or in transient intermediates are originating form ground state weak hydrogen bonds, while the energy released in forming a LBHB is used to help the reaction to be accomplished by lowering its activation barrier (Cleland, 2000).

2.2 Inhibition and enzymatic inactivation

2.2.1 Transition-state analogues

It is generally accepted that the completion of a chemical reaction is passing through unstable short-lived structures, the so-called transition states, whose structures are balanced between ground and product states, while their lifetimes equal approximately that for a single bond vibration (Schramm, 1998). Enzymatic reactions are chemical catalytic reactions which take place in the microenvironment of the enzyme-substrate complex, and hence, our understanding of the enzymatic catalysis should take into account both the structure of the unbound enzyme and its complexes with substrates, inhibitors, intermediates and products; enzymes alter the electronic structure of these latter reactants by protonation, proton abstraction, electron transfer, geometric distortion, hydrophobic partitioning, and interaction with Lewis acids and bases (Schramm, 1998). Herein, we have already commented the substrate binding onto enzymes, as well as the structural role of subsites in the catalysis by specific hydrolases. The transition state inhibitors support the transition state stabilization hypothesis in enzymatic catalysis, and this information helps in comparing transition states, in design transition state inhibitors, as well as in providing a basis for predicting the affinity of enzymatic inhibitors. However, transition state properties cannot always be predicted, as direct information on their structure is available from kinetic isotope effect studies. In this way, enzyme and inhibitor, and/or other transition state analog ligand, should share geometric and electronic similarity as both being necessary in order to provide correct distance to the catalytic site, and to correct hydrogen or ionic and/or hydrophobic bonding in the transition state interactions. The reversible inhibitors are analogs with some minimum structural features of substrates, and thus they get of great theoretical importance in the elucidation of enzyme mechanisms. At least some serious chemical insight into the catalytic mechanism of the enzymatic reaction, and substantial skill, is required for the design and identification of reliable inhibitors. Similar and useful phenomena are the substrate inhibition and activation whose systematic study may identify more pathways and complexes; but it should pointed out that these phenomena are not due to multiple active sites and/or cooperativity effects (Taylor, 2004). In figure 9 are illustrated three hypothetical free energy diagrams corresponding to three distinct cases of reactions, one non-catalyzed and two catalyzed by an enzyme, where $\Delta G^{\ddagger}$, $\Delta G^{\ddagger}_{uncat}$, $\Delta G^{\ddagger}_{cat}$, $\Delta G^{\ddagger}_{M}$, $\mathbf{E}$, $\mathbf{ES}$, and $\mathbf{P}$ are the free energy of activation of an uncatalyzed or catalyzed reaction, their free energy difference, the free enzyme, the enzyme-substrate complex, and the products.
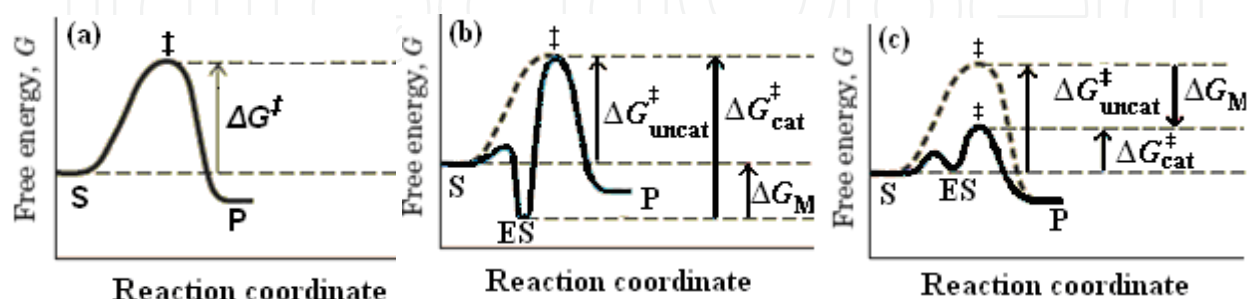

Fig. 9. (a) Non enzymatic reaction, (b) enzymatic reaction where enzyme is complementary to substrate, and (c) enzymatic reaction where enzyme is complementary to transition state. When enzyme is complementary to the substrate (b), the $\mathbf{ES}$ complex is more stable, resulting an increase in the energy of activation (modified from Taylor, 2004).

2.2.2 Stable acyl-enzymes

It has been reported that stable peptidyl acyl-enzymes of elastase may be formed at low pH, while the carbonyl oxygen of the scissile bond occupies the oxyanion hole; moreover, the tetrahedral adduct has a planar structure in the case of ester substrates. The stability of these acyl-enzymes is more likely due to the protonation of H^{57} at low pH-values of the reaction medium (Wilmouth et al, 2001). On the other hand, numerous compounds can be found which inactivate hydrolases through the development of stable acyl-enzyme intermediates; this latter stability is owed to several factors. Firstly, an intrinsic reactivity of the acyl group is experimentally obvious and for ester substrates (especially in proteases and lipases) is reduced due to an increased electron density of the carbonyl group of the scissile bond as substituents become more and more electron-donating; similar effect has been reported in cases where heteroatoms have substituted α-carbons of the amino acid residues of synthetic substrates (e.g. azapeptides) (Gupton et al, 1984). Besides, leaving groups of synthetic substrates, as it is p-nitrophenol could be offset the effects on acylation. A second reason contributing in the stability of acyl-enzyme intermediates is that they do not interact with the oxyanion hole (conversion of $3sp^2$ hybridized carbonyl carbon to $4sp^3$ in the tetrahedral intermediate) (Wilmouth et al, 2001), while a third explanation could be the protonation of H^{57} which disrupts the catalytic triad and cannot activate the nucleophilic water. More reasons of the stability of acyl-enzymes have been reported and they are related to reversible inhibitors, as transition state analogs. More examples could be those reported on the synthesis and the effectiveness of specific peptide reversible inhibitors and/or peptide "sticky" substrates, as useful probes for the investigation of the mechanism of action of particular hydrolytic enzymes (Bieth et al, 1989; Papamichael & Lymperopoulos, 1998).

2.3 Issues in the function of the catalytic motifs

2.3.1 Particular role of several catalytic residues, and one-proton vs. two-proton mechanisms

As we have previously discussed, the active sites of enzymes are equipped with a number of amino acid residuals whose side chains can act as both proton donors and acceptors, allowing proton transfers, and providing catalysis; proton transfers are the most common biochemical reactions. In this section we will be concentrated on the catalytic residues of the enzymes under consideration, and let it be as first example the charge relay system encountered for serine proteases, and under certain circumstances for lipases; the general features of the charge relay system are widely accepted, although the issue of whether the proton is located on the H^{57} or D^{102} (chymotrypsin numbering) has been particularly arguable. Proton transfers have been reported from S^{195} to H^{57} and from H^{57} to D^{102} involving a tetrahedral intermediate formation, as well as neutral D^{102} and H^{57}; this latter requires a two-proton-transfer mechanism which in turn demands that the pK_a of H^{57} should be lower than that of D^{102}, as it is depicted in figure 5(a) (Bieth,1978). In this way, a different mechanism designated as "His flip" has been proposed in an attempt to resolve the problem "one or two protons are transferred" during the acylation process in serine proteases (Bieth, 1989); according to "His flip" mechanism, after the formation of the tetrahedral intermediate, the positively charged histidine flips and place its $N^{\delta 1}$ proton near to the leaving group, although it seems quite unlikely because it violates the principle of least motion (Kidd, 1999). Additional experimental results showed that the charge relay

system operates most likely through the mechanism of figure 4(a,i), in cases of more specific substrates (tetrapeptides or larger) occupying more subsites in the active site of the hydrolase under consideration (Stein et al, 1987; Theodorou et al, 2007a,2007b). Different kinds of ambiguities have been brought up in the case of cysteine proteases, mainly arguing both on the number of catalytic residues, and on how catalysis is accomplished. In conclusion, cysteine proteases are equipped with four catalytic residues, i.e. **C**[25], **H**[159], **D**[158]/**N**[157] and **N**[175] (papain/bromelain numbering - bromelain lacks a **N**[175] residue vs. papain), which may be regarded as a "double catalytic triad", as appeared in figure 4(b). The mechanism of action of cysteine proteases, of the papain family, has been most likely completely elucidated and all uncertainties have been resolved (Theodorou et al, 2001,2007a). The proposed catalytic mechanisms for aspartic proteases comprises two catalytically competent carboxyl groups constituting a functional unit which transfers one proton from the attacking water molecule onto the nitrogen atom of the leaving group. The main issue of these mechanisms requires a coplanar arrangement of the catalytic aspartates where a LBHB joins their $O^{\delta 1}$ oxygens, and it involves proton transfer from the water onto aspartic dyad which is followed by another proton transfer from the dyad onto carbonyl oxygen of the scissile bond; then, the formed intermediate breaks down to products by concerted general acid-base catalysis (Northrop, 2001). Then again in the case of metalloproteases there are certain ambiguities, since two main mechanisms of action have been suggested comprising similarities as well as differences. The similarities include a penta-coordinated Zn^{++} cation, linked with two $H^{\epsilon 2}$-nitrogens and one $P_{1'}$-carbonyl oxygen of the scissile amide bond, while among differences should be mentioned a third $H^{\epsilon 2}$-nitrogen ligand, two oxygen atoms of a catalytic **E**-residue, and that no water is present in the transition state, as they are depicted in details in figure 6. Despite the differences between retaining and inverting mechanisms it is noteworthy that both of them employ a pair of carboxylic acids at the active site with different roles; additionally, both classes of these enzymes operate via transition states with substantial oxocarbenium ion character. A variation on the retaining mechanism involves an ion pair rather than a covalent intermediate (McCarter & Withers, 1994). A general acid, in inverting enzymes, provides one proton for the leaving glycoside oxygen, while a general base supports the nucleophilic attack by a water molecule; on the contrary, in retaining glycosidases a covalent glycosyl intermediate is formed (figure 7).

2.3.2 The hydrolytic water

So far in this text, it has been obvious to a certain extent that water molecules play important roles in the catalysis performed by the hydrolytic enzymes. The hydrolysis of synthetic peptide substrates by serine proteases offers informative examples; the hydrolytic water molecule seems that approaches the acyl-enzyme from the leaving group side and although it should be hydrogen bonded to **H**[57], however it is found in an unfavorable angle relatively to the carbonyl carbon of the scissile bond (Dixon & Matthews, 1989). Inverse solvent isotope effects, found for the reaction governed by the k_{cat}/K_m parameter when several proteases catalyze the hydrolysis of synthetic peptide substrates, seems more likely that they originate from two contributing exchangeable hydrogenic sites in the ground and the transition state. A working example is referred in papain, whose reactive thiolate-imidazolium ion-pair is likely to be in equilibrium with its tautomer neutral thiol-imidazole form, or alternatively, a low barrier hydrogen bond (LBHB) may exist between them; both of

these latter hypotheses are equally likely displaying an effective ground state as a value of $\phi^G = 0.17$ was found by proton inventories (figure 4b) (Theodorou et al, 2001). Similar results were found for the ground state of free enzyme and substrate in case of thiolsubtilisin (Papamichael et al, 2004), and although similar proton inventories were obtained in the case of PPL, in hydrolyzing the ester substrate p-nitrophenyl laurate (L-p-ONP) in aqueous media, however, the value of ϕ^G was found greater than 0.2 arguing for a different way of E-S binding mode. However, in glycosidases the catalytic role of water is profoundly different between retaining and inverting mechanisms, while its effect depends strongly on the ionization of the catalytic acidic residues of the enzyme (figure 7).

3. A step-by-step procedure

3.1 Synthetic part

The art to design and synthesize either a particular single substrate or a series of specific and/or non-specific substrates possessing special binding or interaction properties, it is based on the knowledge of the specificity of subsites, of the enzymes under consideration; as a matter of fact we are dealing with a feedback procedure, where it dominates the logic of "trial and error". In this field of research it could be an advantage the working with proteases; their synthetic substrates are peptides, whose synthesis has found an increased interest due to their huge applications. For example, it has been reported that the active site of papain comprises seven subsites, where the interactions of the $\mathbf{S_1'}$ - $\mathbf{P_1'}$ and $\mathbf{S_2}$ - $\mathbf{P_2}$ character have been found as the more important ones (Kim et al, 1992; Patel et al,1992). Later, by based on the previous experience, the S_1 - P_1 and S_3 - P_3 interactions between purified papain and four newly synthesized peptide substrates were investigated (Papamichael et al, 1999; Theodorou et al, 2001). The logic behind this research was based on the need to elucidate the mechanism of action of cysteine proteases of the papain-C1 family, and although the common substrate Cbz-FR-pNA was convenient for activity assay measurements however, it was experienced as insoluble at higher concentrations so that its Michaelis-Menten parameters were always estimated without reaching the saturation of the used enzyme; the latter is unacceptable by enzyme kineticists, and therefore novel substrates should be synthesized. Finally, we synthesized the substrates X-FY-pNA/ONPh/NMec, where X={Suc, Pht, Cbz} and Y={F, R}. More instructive examples and practices will be provided, herein, which they are referred to different needs and experimental conditions, as well as to the synthesis of both substrates and transition state analog reversible inhibitors for serine proteases (elastase from porcine pancreas – PPE). The conception behind these synthetic probes was based on the need of a serious contribution to the elucidation of mechanism of action of serine proteases; our first results were from suitable ^{13}C NMR studies on the side chain motions of three specifically ^{13}C-labeled trifluoro-acetyl peptide reversible inhibitors bound on PPE (e.g. the $CF_3CO|U-^{13}C|-AA-m-Ph-CF_3$) (Dimicoli et al, 1987). Later, and in this way we synthesized more series of substrates and transition state analog reversible inhibitors for PPE, which were found suitable also, for other serine proteases. Although the most of substrates showed Michaelis-Menten kinetics, however some of them showed substrate inhibition and others substrate activation and along with the synthetic inhibitors led to significant contribution in the elucidation of the mechanism of action of PPE (de la Sierra et al, 1990).

3.2 Computing part: Specific non-linear algorithms and computer programs for experimental data fitting, and suitable tools for statistical analysis (parametric, non-parametric, and experimental design)

It is always the case among enzyme kineticists to have to fit their experimental data with a mostly nonlinear model equation in order to both optimize experimental procedures and to estimate the values of precious parameters (Papamichael, 1987). These procedures may be carried out by using specific nonlinear curve fitting packages, usually equipped with gradient algorithms and requiring from the experimenter a set of initial parameter guessing values; then the package either converges or not, depending on certain factors, whose more important is the continuity of the parameter derivatives, the awkwardness of the model equation, the presence of outliers, and the choice of the fitting algorithm and criterion of convergence (Cornish-Bowden, 1995). In many cases the response of an enzymatic reaction is described by a multi-parametric model-equation which may possesses a more or less awkward character (e.g. multi-substrate kinetics, equations of non competitive and/or substrate inhibition, the Hill equation, etc). Furthermore, the discontinuity of the parameter derivatives of model-equations, which are commonly encountered in enzyme kinetics, is another annoying difficulty, though the relatively high incidence of outliers constitutes a real problem when only few replicates could be obtained as it is the common practice in enzyme kinetic measurements; this latter strongly affects also the choice of the criterion of convergence, as influencing the error distribution (Mannervik, 1982). Therefore, a number of solutions could be suggested to overcome these problems which may include the use of search algorithms instead of the gradients ones, where no need of parameter derivatives is required, as well as non-parametric curve fitting methods where initial parameter guessing values are not required. Likewise, a variety of search algorithms and non-parametric curve fitting methods have been reported, and more or less have been employed successfully (Fletcher, 1965; Papamichael & Evmiridis,1988,2000) on the other hand it is not surprising that enzyme kineticists were involved in such a kind of research trying to provide reasonable solutions to intrinsic problems which often are raised in enzyme kinetics. Independently of the employed algorithm and/or the curve fitting method further robust statistical analysis is necessary for accomplishing a best fit of any nonlinear multi-parametric equation to a series of experimental data (Cleland, 1979). Thus, three additional issues should be taken into account namely the errorless and unbiased estimation of the involved parameter values, as well as the application of suitable information criteria for the discrimination among model-equations, which in several situations employ the same number of parameters (Cleland,1979); the third issue is due to the inborn problem of enzyme kinetics where statistically few experimental data supplied with few replicates impose for an optimal experimental design in order to minimize error and maximize the precision of the parameter estimates (Box, 1971, Kafarov, 1976). Then again, a variety of relative works and methods may be suggested whose application will surmount these three additional requirements (Evmiridis &. Papamichael, 1991; Papamichael, 1995; Comish-Bowden & Eisenthal, 1974).

3.3 Experimental – Kinetic part

3.3.1 Evidence for Michaelis-Menten and/or other type of kinetic behavior

Mathematical model-equations along with computer techniques are valuable tools in searching for optimal experimental conditions and effective enzymatic action. The study of

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various model-equations incorporates the information concerning each particular enzymatic reaction or system, it attains of great interest in the basic research and applications of these biocatalysts and they have been proved as effective tools in estimating the process variables. The rate equation of an enzymatic reaction illustrates the catalytic process in terms of rate constants and reactant concentrations; the initial rate of an enzymatic reaction is directly proportional to the concentration of enzyme preparation, and it is increased nonlinearly with increasing the substrate concentration up to a limiting maximum value. The well know Henri-Michaelis-Menten equation (1) is based on reasonable assumptions associated with the quasi-steady-state approximation (QSSA) (Michaelis & Menten, 1913), where the relations *[E]ₜ=[E] + [ES]* and *[S]ₜ ≈ [S]* are valid (*[E]ₜ, [E], [S]ₜ,* and *[S],* are the total and free enzyme, and the total and free substrate concentrations, respectively); the physical meaning of its two parameters V_{max} and K_m is familiar among enzymologist. Currently, any non-linear model equation as the Henri-Michaelis-Menten (H-M-M) one can be used for fitting enzymatic experimental data and obtaining parameter estimates due to the available computers and software. Not often, the H-M-M equation cannot fit experimental data from enzymatic reactions; then, it is not uncommon to assume some reasonable modifications of equation (1) to succeed the best fit. It is recommended to use rational equations having the general form of equation (2), where **n ≥ m**, whereas the meaning of parameters **a₁, a₂,…aₙ,** and **b₁, b₂,…bₘ,** depends on the reaction itself and the experimental conditions (NC-IUB, 1983). A specific case of equation (2) is equation (3) known as the Hill equation (Hill, 1913), and it is valid when polymeric enzymes extend positive cooperativity, consisting of more than one subunits, comprising **n** equivalent binding sites (a single substrate molecule is bound per subunit). In equation (3), the parameter K_H no longer equals the substrate concentration that yields half-maximal velocity except for **n = 1**. In equation (3) **α = - ln(K_H)**, **β = n** (**n** is the Hill coefficient and K_H is the Hill parameter which is similar to K_m), and **x = ln(*[S]*)**.

$$v = \frac{k_p [E]_t [S]}{K_S + [S]} = \frac{k_p [E]_t [S]}{[S] + \frac{(k_{-1} + k_p)}{k_1}} = \frac{V_{max} [S]}{[S] + K_m} \tag{1}$$

$$v = \frac{a_1[S] + a_2[S]^2 + a_3[S]^3 + ... + a_n[S]^n}{1 + b_1[S] + b_2[S]^2 + b_3[S]^3 + ... + b_m[S]^m} \tag{2}$$

$$v = \frac{V_{max} [S]^n}{K_H + [S]^n} = \frac{V_{max} \frac{[S]^n}{K_H}}{1 + \frac{[S]^n}{K_H}} = \frac{V_{max} e^{a + \beta x}}{1 + e^{a + \beta x}}, \text{ and } \ln[\frac{[S]^n}{K_H}] = n\ln[S] - \ln(K_H) = a + \beta x \tag{3}$$

The rate of any reaction, including the enzymatic ones, depends on the number of contacts between the different kinds of molecules, and in isotropic systems is proportional to the product of concentrations of reactants. When the reactive species belong to macromolecular systems, as they are the enzyme molecules, the evaluation of the average number of their

contacts should take into account all different conformations of these macromolecules. This latter is rarely taken into account in enzymatic reactions, which are handled as homogeneous because experimenters use to keep always valid the relation $[S]_t \gg [E]_t$ (Segel, 1975). In this way, and in order to describe different behaviors in enzyme kinetics, a variety of multi-parametric nonlinear equations have been proposed whose parameters are surprisingly complicated functions of individual rate constants. Attempts to counterbalance this situation led a number of authors to present alternative mathematical formulations in enzyme kinetics; characteristic examples may be equations (4) and (5), where D, V^{eff}_{max} and K^{eff}_m are the fractal dimension and the effective individual H-M-M parameters, while A_2, A_3, etc. are the equivalent virial coefficients, respectively (Lymperopoulos et al., 1998; Savageau, 1998). Equations (4) and (5) offer appreciable economy in numerical treatment of enzyme kinetic experimental data, as compared with the conventional equations, allowing an overall view of the complexity of the reaction path of enzymatic catalysis; when $D = 1$ equation (4) takes on the form of H-M-M equation, and V^{eff}_{max}, and K^{eff}_m receive their ordinary meaning. In the case of equation (5) we should recall the QSSA condition on which it was based on the development of the H-M-M equation and where $\left(-\dfrac{d[ES]}{dt}\right) = (k_{-1} + k_p)[ES] = \left(+\dfrac{d[ES]}{dt}\right) = k_1[E][S]$; instead, it can be written that $k_1[E][S] = C(E,S)$ i.e. the average number of contacts between the two reactants, which in turn depends on the kind of more complicated contacts between **S** and **E**, and leads to a complicated dependence on **[S]**, i.e. $C(E,S) = [E][S](1 + A_2[S] + A_3[S]^2 + A_4[S]^3 + \ldots)$. Moreover, virial coefficients can be positive or negative expressing positive or negative contributions to $C(E,S)$ from pairs, triples etc. of **S** in the neighborhood of **E**. Both equations (4) and (5) can fit a variety of experimental data (Lymperopoulos et al., 1998; Savageau, 1998).

$$v = \frac{V^{eff}_{max}\,[S]^{2-D}}{[S] + K^{eff}_m} \tag{4}$$

$$v = \frac{V_{max}\,[S](1 + A_2[S] + A_3[S]^2 + \ldots)}{K_m + [S](1 + A_2[S] + A_3[S]^2 + \ldots)} \tag{5}$$

3.3.2 Experimental design – Factorial experimentation

In a previous section we referred and explained a number of reasons on the necessity of use the experimental design, as an essential method for the minimization of time, cost, and the wasting of valuable and expensive reagents; now we ought to describe how this could be done in the course of enzyme kinetics. Any general procedure in obtaining experimental

design for nonlinear multi-parametric equations should take into account the range where its independent variable is defined, as well as the insurmountable occurrence of experimental errors, and it comprises the following: (a) establishment of a model-equation which best fits the experimental points, (b) choice of the optimality principle, (c) reasonable parameter estimates for the model-equation, (d) validation of the performance of the optimal-design experimental points. Herein, we will adopt the **D**-optimality principle in finding the required optimal experimental points and obtain parameter estimates of the chosen model-equation with the maximum possible precision. We will focused to multi-parametric nonlinear model equations with one independent variable i.e. $y = f(x; \theta_1, \theta_2 \ldots \theta_r) = f(x; \theta_p)$ with p parameters ($p = 1, 2 \ldots r$), where $\theta_1, \theta_2 \ldots \theta_r$ are the parameter estimates, while the experimental series are defined by the column vector $D = \{x_i\}$. Then, we have to choose a set of $\mathbf{x_i}$ values ($i = 1, 2 \ldots n$), in order to observe the response y; so that from these observations to estimate the p parameters as precisely as possible; n is the number of trials, i.e. the optimal-design experimental points. In such a case, for single independent variable equations, the column vector $D = \{x_i\}$ is identical with the design matrix which is replaced by the *Jacobian matrix* **F*** for nonlinear multiparametric model-equations (Papamichael et al, 1995). The partial derivatives of a multi-parametric nonlinear model-equation with respect to the $\mathbf{r^{th}}$ parameter θ_r for each

optimal-design experimental x_i point are $f_{ir}^* = \dfrac{\partial f(x_i; \theta)}{\partial \theta_r}$, and $\underline{\theta} = \begin{bmatrix} 1^* \\ 2^* \\ . \\ r^* \end{bmatrix}$ is a set of

parameters obtained by preliminary curve fitting ($i = 1, 2 \ldots n$, and $r = 1, 2, \ldots r$), and considered to be very close to the true parameters. The $n \times p$ dimensional matrix $\mathbf{F^*} = \{f_{ir}^*\}$ is usually chosen to have $n = p$ and accordingly the relation $\left| F^{*T} \bullet F^* \right| = \left| F^* \right|^2$ is valid; then, the procedure is an attempt to maximize the modulus of the determinant $\left| F^* \right|$, or to minimize its inverse $1/\left| F^* \right|$, while when $n > p$ the alternative is to maximize $\left| F^{*T} \bullet \mathbf{F}^* \right|$ or to minimize its inverse. The experimenter should proceed by drawing a diagram having common abscissa the independent variable of its model-equation, and as ordinates the partial derivatives of the model-equation with respect to each single parameter, i.e. $\partial \mathbf{y}/\partial \theta_1, \partial \mathbf{y}/\partial \theta_2, \ldots \partial \mathbf{y}/\partial \theta_r$; then, as optimal points are selected these ones where the abscissa values of the partial derivatives present minima or maxima, or even approached and/or are removed each other. Furthermore, it is recommended to ultimate the optimization studies by taking into account both cases $n = p$ and $n > p$, and then to consult the inverse correlation ($\mathbf{R^{-1}}$) and the hat (**H**) matrices (Papamichael et al, 1995).

There are many procedures, where the experimenter is concerned with the effect of a series of parameters on certain estimated attributes. In such cases factorial analysis is a key method, where "factors" are variable parameters to be considered in the experiment, and are distinguished in either quantitative (varied continuously) or qualitative (cannot be

varied continuously) ones. Typically, the experimenter, should assign the values of quantitative factors at pre-determined levels (e.g. low, medium, high), while this is not requisite for the qualitative factors. The collection of levels of all employed factors in a given trial has been designated as the "treatment"; this latter provides a full description of the experimental conditions which affect the studied factors and where the term "experiment" refers to the whole collection and not to an individual trial. Then, the numerical result per trial, based on a given treatment, has been designated as "response", which is the attribute we measure. Normally, the value of a response is varied as the factor level is varied, whereas factors are regarded as independent variables and responses as dependent variables. In order to determine the effects of one or more factors on a specific chemical feature we should carry out one or more experimental trials with each of the possible combinations of the levels of the factors, and that is the so-called "factorial experiment". Factorial experiments may be performed from the simplest case at two levels up to more composite and of higher difficulty combinations. A factorial experiment at two levels may involving two factors A (e.g. temperature) and B (e.g. feed rate of a reactor). In order to find the effect of A on B, a first trial with A at T_1=50ºC and B at Fr_1 = 50 ml/h, and a second trial with A at T_2=150ºC and B at Fr_2 = 75 ml/h, were performed to find the effect of temperature; then a third trial at T_1=50ºC and B at Fr_2 = 75 ml/h as well as a fourth trial at T_2=150ºC and Fr_2 = 75 ml/h were performed in order to find the effect of the feed rate. From these data it could be defined an effect due to variation in factor A as follows i.e. effect of A = (33 - 27 = 6), and effect of B = (8 - 27 = -19). In each of these effects they were used only two of the three observations, and while the observation T_1=50ºC and Fr_1 = 50 ml/h was used twice, while the rest two observations were used only once. Moreover, by varying one factor at a time we cannot comment the possibility of interaction between these two factors; the fourth trial may give the solution. Both A and B should be increased for higher yield, i.e. the effect of A at B-first level equals to 33 - 27 = 6, and the effect of A at B-second level = 70 - 8 = 62 (Davies & Henrissat, 1995).

3.3.3 Detailed analysis of the experimental data from pH and temperature profiles, of the Michaelis-Menten parameters, and estimation of useful constants and/or relations

It is common to assign pK_a values on free enzyme (**E**) from k_{cat}/K_m vs pH profiles, by based on the ambiguous assumptions that proton transfers to and from **E** and **S** are much faster than formation and breakdown of the **E-S** complex; similarly common is to assume that productive binding is allowed only to a single protonic state of the enzyme. However, both assumptions are valid in cases of sticky substrates i.e. in cases where substrates dissociate from the active site of enzyme at a rate comparable to or slower than that at which the **ES** complex reacts to form product (Cleland, 1977); the stickiness of a substrate is proportional to the ratio k_2/k_{-1} (Theodorou et al, 2001). Nevertheless, in a general point of view the profiles of the dependencies of Michaelis-Menten parameters vs. pH show many reactive hydronic states, comprising equal number of pK_a values and equal or less number of pH-independent rate constants. In order to determine such parameters as they are the values of pK_as and of rate constants, it is necessary to plan and employ appropriate equations derived by using models in which enzyme and substrate ionizations are considered to be at QSSA (Brocklehurst et al, 1979). The following reaction figures 10 along with equation (6) it may be

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an instructive example. However, the experimental data from Michaelis-Menten parameters vs. pH dependencies profiles should normally described by the general equation (7), where k_{obs} is the estimated value of the rate constant, $(k)^{lim}$ is its marginal maximum value for the hydronic state EH_{i-1}, *n* is the number of reactive hydronic states, B_{ij} is a description of the form $K_{XHp}[H^+]^m$, while *m* and *p* are elements of two matrices **I** and **II**, corresponding to *i* and *j* indices, respectively.
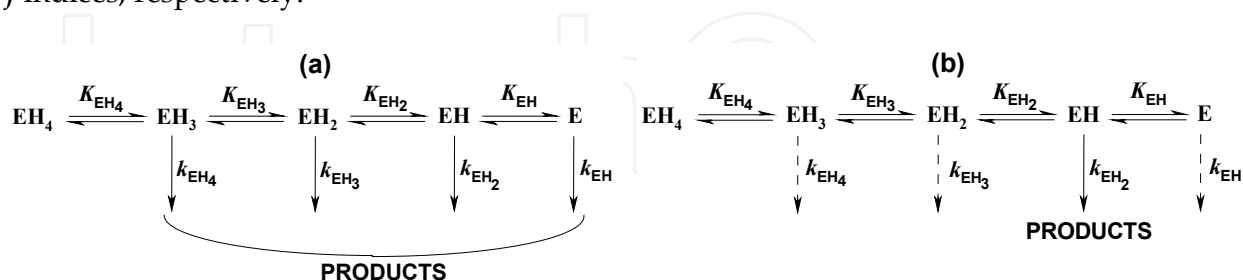
Fig. 10. (a) reaction scheme involving four reactive hydronic states from **E** to **EH₃** and one **EH₄** not reactive, characterized by four macroscopic acid dissociation constants and four pH-independent rate constants, (b) similar reaction scheme where only one hydronic state designated as **EH** is reactive and characterized by four macroscopic acid dissociation constants and only one pH-independent rate constant; this latter is a particular case of (a).

$$k_{obs} = \frac{(k_{EH})^{lim}}{1 + 10^{pK_{EH_4}+pK_{EH_3}+pK_{EH_2}-3pH} + 10^{pK_{EH_3}+pK_{EH_2}-2pH} + 10^{pK_{EH_2}-pH} + 10^{pH-pK_{EH}}} \quad (6)$$

$$k_{obs} = \sum_{i=1}^{n} \frac{k_i^{lim}}{\left(1 + \sum_{j=1}^{n} B_{ij}\right)} \quad (7)$$

Matrix I

	j=1	2	3	4	5	6...n
i=1	1	2	3	4	5	6...n
2	-1	1	2	3	4	5...n-1
3	-2	-1	1	2	3	4...n-2
4	-3	-2	-1	1	2	3...n-3
5	-4	-3	-2	-1	1	2...n-4
6	-5	-4	-3	-2	-1	1...n-5
...	...	...	...	...	...	...
n	1-n	2-n	3-n	4-n	5-n	6-n...1

Matrix II

	j=1	2	3	4	5	...n
i=1	-1	-1.2	-1.2.3	-1.2.3.4	-1.2.3.4.5	...-1.2.3...n
2	1	-2	-2.3	-2.3.4	-2.3.4.5	...-2.3.4...n
3	1.2	2	-3	-3.4	-3.4.5	...-3.4.5...n
4	1.2.3	2.3	3	-4	-4.5	...-4.5.6...n
5	1.2.3.4	2.3.4	3.4	4	-5	...-5.6.7...n
6	1.2.3.4.5	2.3.4.5	3.4.5	4.5	5	...-6.7.8...n
...	...	...	...	...	...	...
n	1.2.3..n	2.3.4..n	3.4.5..n	4.5.6..n	5.6.7..n	...-n

The thermal variation of enzymatic reaction rate constants conform to the well known Arrhenius equation: $k = A\, e^{-E/RT}$, where *A*, **E**, **R** and **T** represent the frequency factor, the activation energy, the gas constant and the absolute temperature, respectively; *k* may be any one of the Michaelis-Menten and/or other enzymatic mechanism rate constants, and thus its value could be generally evaluated. The thermo stability of enzymatic activity is a prerequisite and it should be tested by incubating enzyme preparations for sufficient time at as possible high and/or low temperatures, and then by measuring their activity at the optimum

temperature (Papamichael, & Theodorou, 2010). By integrating the Arrhenius equation, the temperature dependence of enzymatic rate constants can be found and illustrated in equation (8), assuming constant activation energy; in equation (9) k_j, $k_{j,0}$, E_j, R, T_0 and T, are the rate constant under consideration at different temperatures, its value at the reference absolute temperature, the activation energy associated with the rate constant, the gas constant, and the reference absolute temperature, and the independent variable of equation (Papamichael et al, 2010). When k_j equals to either k_{cat}/K_m or k_{cat}, then equation (8) is transformed to equation (9) or (10), where $E_a=E_{-1}-E_2$, and $a=k_2/k_{-1}$, and are referred to the well known "three-step

mechanism": $$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E S \xrightarrow{k_2} E\text{-}S^* \xrightarrow{k_3} E + P$$ (Wang et al, 2006). In

homogeneous reactions and in terms of the transition state theory (TS) the activation energy $E_a = \Delta H^{\ddagger} + RT$ can be evaluated as it has been formulated by the Eyring equation ($k = (k_B/\hbar)Te^{-\Delta G^{\ddagger}/RT} = (k_B/\hbar)T\ e^{-\Delta H^{\ddagger}/RT}\ e^{-\Delta S^{\ddagger}/R}$). Therefore, the study of rate constants as function of the absolute temperature provides thermodynamic data on the TS (Papamichael, & Theodorou, 2010).

$$k_j = k_{j,0}\ \mathrm{Exp}\left[-\frac{E_j}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right]$$ (8)

$$\frac{k_{cat}}{K_m} = \frac{k_{1,0}\ a_0 \mathrm{Exp}\left[\dfrac{E_a}{R}\left(\dfrac{1}{T} - \dfrac{1}{T_0}\right)\right]}{1 + a_0 \mathrm{Exp}\left[\dfrac{E_a}{R}\left(\dfrac{1}{T} - \dfrac{1}{T_0}\right)\right]}\ \mathrm{Exp}\left[-\frac{E_1}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right]$$ (9)

$$k_{cat} = \frac{k_{2,0}\ k_{3,0} \mathrm{Exp}\left[-\dfrac{E_2 + E_3}{R}\left(\dfrac{1}{T} - \dfrac{1}{T_0}\right)\right]}{k_{2,0}\ \mathrm{Exp}\left[-\dfrac{E_2}{R}\left(\dfrac{1}{T} - \dfrac{1}{T_0}\right)\right] + k_{3,0} \mathrm{Exp}\left[-\dfrac{E_3}{R}\left(\dfrac{1}{T} - \dfrac{1}{T_0}\right)\right]}$$ (10)

4. Application of several effective methods and tools

4.1 Kinetic and thermodynamic methods

4.1.1 H/D solvent isotope effects, and proton inventories, on constants and parameters

The light hydrogen isotope protium (H) can be replaced by deuterium (D) in the hydrogenic sites of the water species (i.e. HOD, D_2O, H^+_3O, H^+_2OD, D^+_2OH, D^+_3O, etc), under certain circumstances, and as a consequence deuterium may replace protium into some sensitive positions of enzymes and substrates; these replacements have been designated as solvent isotope effects (SIE) and usually they affect the kinetic and equilibrium constants associated with the enzymatic reactions. Obviously, these SIE are related to the isotopic solvents and thus

should disturb rate and/or equilibrium constants, and influence both initial (ground) and final (transition or product) states. In the course of the development of an enzymatic reaction mechanism, it is commonly required to identify isotope effects for individual hydrogenic sites, where the notion of the isotopic fractionation factors is used. Furthermore, the hydrogenic sites are classified into these established in the structural framework of the solutes (internal ϕ-sites), as well as to those found in the isotopic water molecules (external ϕ-sites) which strongly interact with the solutes. It is a mechanistic affair to distinguish between ϕ-sites and it is based on information arose from solvent isotope-effect data. Based on the above concepts new extra methods can be originated, which are useful in enzyme kineticists. One of them is the "proton inventories", which seems more likely that they constitute reliable tools in investigating enzymatic mechanisms (Theodorou et al, 2007b); this method comprises kinetic studies of SIE in a series of mixtures of H_2O and D_2O, while the reaction parameters are expressed as $k_n(n)$ functions of deuterium atom fraction $n = [D_2O]/\{ [H_2O]+[D_2O]\}$ present in the isotopic solvent, according to equation (11). In equation (11) k_0 is a reaction rate in H_2O, and ϕ_i^T and ϕ_j^G are the isotopic fractionation factors of **i**th transition state, and of **j**th ground state protons, respectively; the values ϕs, as well as the shape of the $k_n(n)$ functions, and the number of the transferred protons are diagnostic of the reaction mechanism (Theodorou et al, 2001). Gross and co-workers had published a series of papers on phenomena in mixtures of isotopic solvents, where they introduced a critical assumption for the easy use of solvent isotope effects. Accordingly, the relative abundances of all probable species in isotopic waters could be determined solely by statistics and by the ratio of protium to deuterium in the medium, with the additional assumption that deuterium rather than protium occupies the hydronic sites of enzyme and/or substrate independently of whether deuterium or protium is already found onto that site. This assumption is currently known as the Rule of the Geometric Mean, and led both Gross and Butler to a particularly clear algebraic formulation of the dependence of the reaction rate on **n**. To determine the significance of parameters of the Gross-Butler-Kresge equation (11), all previous theoretical approaches should be used in order to formulate simplified forms of this equation capable to best fit the available series of the experimental data; a variety of simplified forms of equation (11) have been already published and can be easily incorporated into any curve fitting program and/or algorithm.

$$k_n = k_0 \frac{\prod_{i=1}^{\mu} (1 - n + n\phi_i^T)}{\prod_{j=1}^{\nu} (1 - n + n\phi_j^G)} \tag{11}$$

4.1.2 Determination of kinetic isotope effects via NMR studies ([1]H v.s. ^{2}H NMR spectroscopy and NMR-Proton inventories for intramolecular/intermolecular hydrogen transfer reactions)

NMR spectroscopy is one among many probes that have been reported for the evaluation of kinetic isotope effects. Several NMR methods, as analogs of previous proton inventory techniques involving classical kinetic methods were reported, involving line-shape analyses and polarization transfer experiments on the exchanging protons or deuterons and/or on the remote spins as functions of the deuterium atom fraction **n** in the mobile proton sites. Moreover, the kinetic isotope effects and the number of transferred protons originating from

the NMR spectra were developed both in theory and praxis by the application of a specific technique. The theory was based on the study of kinetic isotopic exchange reactions (e.g. for two molecules **AH** and **AD**, there is the option of performing experiments either on the **A** spins, the **H** and/or **D** spins), where the obtained various experimental kinetic quantities depend on which nuclear spins are chosen for the experiments. In following, the relation between rate constants of intermolecular H/D transfer reactions and inverse lifetimes measured by NMR spectroscopy were estimated. These theoretical description may be more comprehensible if the features of NMR spectroscopy will be demonstrated by considering a simple equilibrium $i \rightleftharpoons j$, which is described by the constant $K_{ij} = k_{ij}/k_{ji} = [j]/[i]$. Generally, any NMR experiment is performed under equilibrium, and the NMR line-shapes depend on the inverse lifetimes, according to equation (12), where $d[i{\rightarrow}j]/dt$ is the number of molecules per volume leaving from the environment i to j during time **dt**. A first approach to a more skilled and profound knowledge on these methods and techniques is the excellent work of H-H. Meschede and Limbach (1991), where is given a theoretical background of dynamic NMR spectroscopy in the presence of kinetic isotope effects, as well as applications and selected examples (Meschede & Limbach, 1991).

$$\tau_{ij}^{-1} = k_{ij.} = -[i]^{-1} \times d[i{\rightarrow}j]/dt \qquad (12)$$

4.1.3 CD-stopped flow spectroscopy

In order to examine early events during a reaction it is necessary to employ rapid mixing and detection system, such as that provided by stopped-flow apparatus including the stopped flow CD techniques and devices. Moreover, stopped flow CD has been used extensively in kinetic measurements of conformational transitions in proteins and in protein folding studies on a millisecond time scale, allowing the collection of time-resolved CD spectra. The changes in ellipticity in both the far and near UV give information about the regain of secondary and tertiary structure respectively, providing valuable information on the mechanism of protein folding (Kelly & Price, 2000). The limitation of stopped-flow CD is the "dead-time" of the mixing process which is typically almost more than 2 milliseconds (Clarke & Jones, 1999).

4.1.4 Substrate assisted catalysis

The classic is that all necessary groups which are essential for catalysis are provided by the enzyme to convert substrates into products; however, there are some exceptions in this rule, where the substrate may provide also functional groups which will participate in the catalytic reaction course. This phenomenon has been reported in either case of native enzymes or engineered mutants, and it is designated as "substrate-assisted catalysis" (SAC). Several enzymes namely serine proteases, GTPases, type II restriction endonucleases, lysozyme and hexose-1-phosphate uridylyl-transferase have been shown as susceptive for substrate assisted catalysis. As examples of SAC in naturally occurring enzymes may be the type II restriction endonucleases, while examples of engineered enzymes may be serine proteases; in that latter case a functional group from a substrate can substitute a catalytic

residue of enzyme which has been replaced by site-directed mutagenesis. SAC, in serine proteases, is mainly used in order to modify specific sites for proteolytic cleavage, and it may provide strategies for change of the catalyzed reaction. In the point of view of an enzymologist, SAC may contribute significantly to the activity of some enzymes representing functional intermediates in the evolution of catalysis. In figure 11 is given an example of substrate assisted catalysis (Dall'Acqua & Carter, 2000).
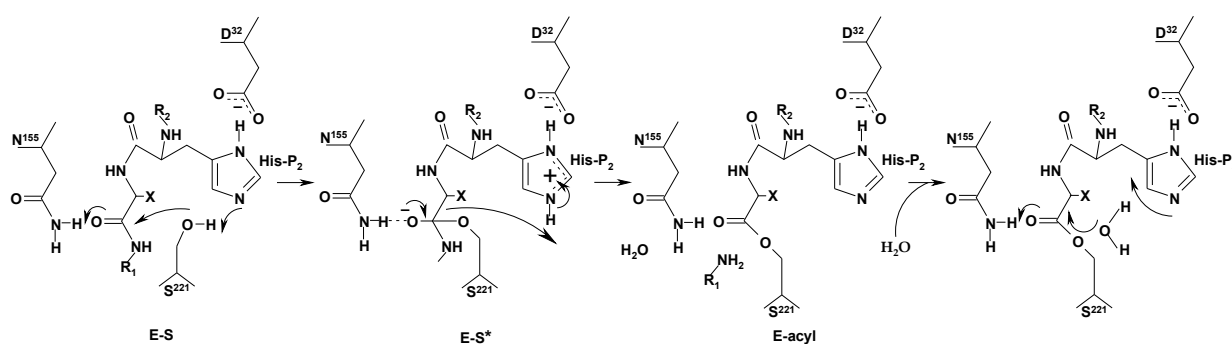

Fig. 11. The H64A subtilisin BPN9 mutant with a peptide substrate containing a histidine residue at the P_2 position, where the mutated catalytic residue has been omitted for clarity (modified from Dall'Acqua & Carter, 2000).

4.1.5 Detecting the rate-limiting step (R.L.S.) and identifying the kinetic (hydrolytic) mechanism

Usually the terms (a) rate-limiting step, (b) rate-determining step, and (c) rate-controlling step are regarded as synonymous; however, other meanings that have been given to them should be mentioned, as it is necessary to be aware of them in order to avoid confusion. The term rate-determining step is mostly used as special case of rate-controlling step, being assigned only to an initial slow step which is followed by rapid steps. Therefore, in an enzymatic reaction, the rate-limiting step is generally the stage that requires the greatest activation energy or the transition state of highest free energy. The idea of a rate-limiting step is a fundamental concept for understanding reaction rates. Thus, the slowest step of a multi-step reaction is often called the rate-limiting or rate-determining step. An approach to finding the rate-limiting step is based on locating the transition state of highest energy. However, this method may fail for irreversible reactions where intermediates more stable than the reactants usually occur; another approach which has been used in the kinetic mechanism of cysteine proteases, and it could found more general usages, is the application of the PI method along with pH and absolute temperature profiles in a attempt to estimate the individual rate constants.

4.1.6 Distinguishing between concerted and stepwise mechanisms

In this section we would like to approach what it concerns the general acid-base catalysis and its associated proton transfer. For example, what determines whether a reaction proceeds by stepwise acid-base catalyzed or concerted reaction mechanisms?, or does catalysis take place in such a way as to avoid the most unstable intermediate?, etc. Answers to these queries may be found in a rule which states that: "Concerted general acid-base

catalysis of complex reactions in aqueous solution can occur only (a) at sites that undergo a large change in pK_a in the course of the reaction, and (b) when this change in pK_a converts an unfavorable to a favorable proton transfer with respect to the catalyst; i.e., the pK_a of the catalyst is intermediate between the initial and final pK_a values of the substrate site"; however, "the rule does not apply to certain diffusion-controlled reactions in which separate proton transfer steps are not possible. It does apply to the separate steps of reactions proceeding through intermediates so long as these intermediates have a significant lifetime; if there are no such intermediates it should be applied to the overall reaction" (Jencks, 1972). It is commonly the case that a reported enzymatic mechanism seems more or less as incomplete in that the structures of its transition states are not defined in terms of bond orders, atomic charges and/or other features. This can be achieved by specifying the degree of proton transfer (PT), from a nucleophilic reagent onto an electrophilic one, as well as the degree of heavy atom reorganization (HAR); this latter corresponds to the extent of bond breaking and/or making to heavy atoms. Usually, a transition state structure could be described by a diagram of two dimensions relating HAR to PT. Therefore, appropriate experimental data from proton inventories and rate constant vs. pH profiles are prerequisite in order to draw a HAR/PT diagram for enzymatic reactions proceeding via the formation of tetrahedral intermediates. Besides, some reports disagreed with the above rule of Jencks, based on the Marcus theory (Marcus, 1965) insisting that the transition state for proton transfer reactions occurs at a value of HAR such that the free energy change for proton transfer is zero. This condition is satisfied when the pK_a values of the proton-accepting and proton-donating species are equal (Szawelski, 1981). An illustrative example in distinguishing between concerted and/or stepwise character may be the identification of the mechanism of action of cysteine proteases of the papain C1 family, where a large number of experimental works have contributed a lot in this latter course; however the application of the proton inventory methodology (PI) along with the use of k_{cat}/K_m and k_{cat} vs. pH profiles were shown as decisive and reliable tools in investigating enzymatic mechanisms. In more details and for the hydrolysis of specific substrates by papain, chymopapain and stem bromelain, were estimated the values of the Michaelis-Menten parameters (k_{cat}/K_m, k_{cat}, K_S), the values of other rate constants (k_1, k_{-1}, k_2, k_3) as well as the values of all related pK_as and the corresponding fractionation factors and SIE, from PI studies, and kinetic parameters/rate constant vs. pH profiles (Ishikawa, 2007; Pavlovsky,1999; Theodorou, 2001,2007a). Therefore, by taking into account results like these described above, HAR/PT (heavy atom reorganization/proton transfer) diagrams could be constructed (figure 12); the examples which are given below are referred in the hydrolysis of p-nitroanilide substrates by cysteine proteases of the papain family.

4.2 Particular NMR-spectroscopy methods

4.2.1 Liquid-state NMR spectroscopy: Hydrogen NMR spectroscopy (^{1}H, ^{2}H, and ^{3}H), Heteronuclear NMR spectroscopy (^{13}C, ^{15}N, ^{18}O, and ^{19}F), studies on enzyme dynamics

Both nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography are the only biophysical methods which can provide high-resolution structures of biological molecules such as proteins and their complexes at atomic resolution. Additional information can also be provided about conformational dynamics and exchange processes of
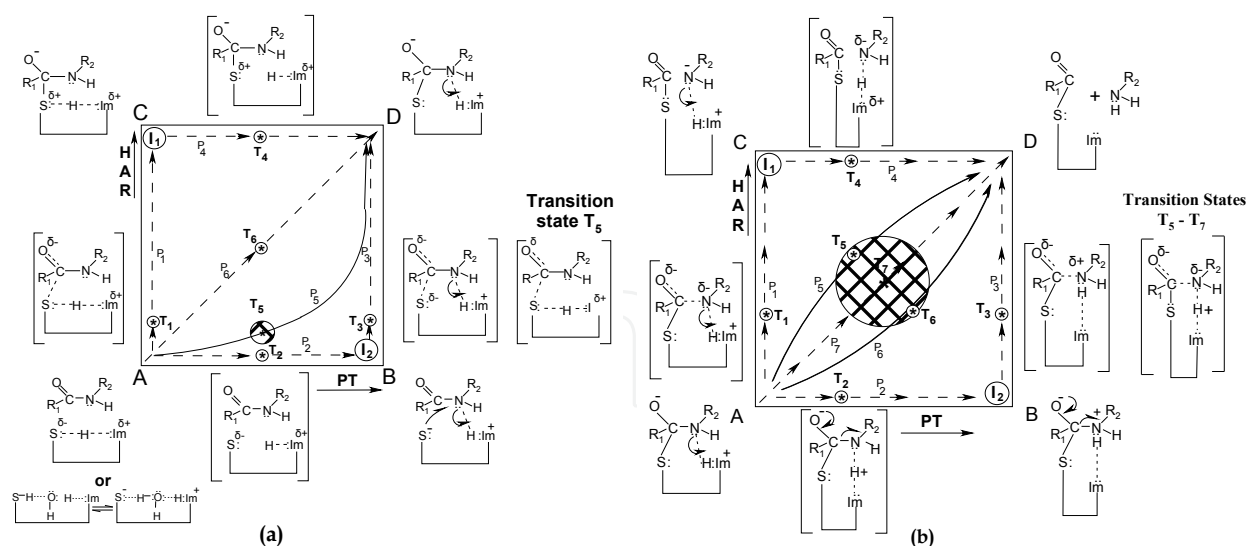
Fig. 12. HAR/PT diagrams where **A** symbolizes the reactants and **D** the products: (a) the boxed diagram represents the most probable transition state T_5, corresponding to a stepwise pathway P_5, (b) the boxed diagram represents the most probable transition states T_5 to T_7, corresponding to the concerted pathways P_5 to P_7.

biomolecules at timescales ranging from picoseconds to seconds, and is very efficient in kinetics of enzymatic reactions (Torchia & Ishima, 2003). NMR results from the absorption of energy by a nucleus changing its spin orientation in a magnetic field. Protons (^1H) are the most commonly studied nuclei; because of high natural abundance (99.9885%) and high gyromagnetic ratio; their resonance spectrum is characteristic of the various groups in the molecule. Modern NMR goes far beyond analysis of groups. Two types of NMR interactions through-bond spin-spin and the through-space nuclear Overhauser effects can be used to determine the three-dimensional structure and dynamics of macromolecules in solution (van Holde et al., 2006). Moreover, the deuterium nucleus has a spin of 1, resulting in three energy levels in a magnetic field and a non-zero quadrupole moment, and has found a wide use. Deuterium NMR spectroscopy in solutions yields spectra that are similar to proton NMR spectra except that the deuterium peaks are slightly broader due to quadrupole interactions. Liquid-state deuterium NMR has been used extensively to monitor deuterium labeled species in kinetic and mechanistic studies (van Holde, 2006). For historical and practical reasons, a differentiation between proton and heteronuclear NMR was generally taken into account. However, this distinction is not justified regarding chemical shift and spin coupling, as the basics of NMR spectroscopy are identical for all nuclei. In practice, a differentiation is reasonable for a few important principles i.e. the occurrence of nuclei (Holzgrabe,2008). Nowadays, most successful applications of NMR to biological systems have been typically carried out in aqueous solutions, and have utilized spin $I = 1/2$ nuclei, such as ^1H, ^{13}C, ^{15}N and ^{31}P and others, whereas NMR spectroscopy has proved less powerful when applied to nuclei with spin quantum number $I >> 1/2$, i.e. those having a non-spherical charge distribution and an electric quadrupole moment. Unfortunately, for a large number of biologically relevant elements, the only NMR-active isotopes are those with nuclear spins greater than ½ (Ronconi & Sadler 2008). The study of ^{13}C nuclei through NMR spectroscopy is an important technique for determining the number of nonequivalent carbons and to identify the types of carbon atoms that may be present in a compound (Pavia

et al., 2009). A complication in using ^{13}C in NMR spectroscopy was the occurrence of ^{13}C-^{1}H coupling involving the many protons normally present in organic compounds; this latter problem was solved by the development of wide band proton decoupling. It is often necessary to prepare compounds enriched in ^{13}C beyond the natural abundance. The ^{17}O is the only NMR-active oxygen isotope (*I = 5/2*) and its application in NMR has been hindered by several of its intrinsic nuclear properties resulting in low sensitivity and complex spectra; however, ^{17}O NMR spectroscopy in solutions is a useful technique to solve structural problems for small organic molecules. The large quadrupolar interaction of oxygen-containing functional groups can cause highly effective relaxation, which leads to strong broadening of the NMR signals, which can be severe for large molecules. On the other hand, the ^{19}F has many favorable NMR characteristics, including a nuclear spin *I = 1/2*, 100% natural abundance, high sensitivity and large chemical shift range (<500 ppm) (Battiste & Newmark, 2006). Endogenous fluorine has a very short T_2 relaxation time and the resulting signal is below the limits of NMR detection in most biological systems of interest (Yu et al., 2005). The ^{15}N Nitrogen nuclei are frequently located at the interaction sites of biomolecules; for example, amide nitrogens in peptides are the key in maintaining the peptide backbone conformation by hydrogen bonding. The side chains of amino acids H, W, and R contain nitrogen atoms which are often located at the active sites of enzymes. To detect interaction sites and to study the interaction mechanism of these biomolecules, the use of nitrogen NMR seems promising (Inomata et al, 2009). The natural abundance of ^{15}N nuclei with a spin quantum number I = 1/2 (which give a sharp resonance signal) is only 0.3%. But recent developments in the instrumentation of NMR spectroscopy have made it possible to observe the resonance of the nuclei with low natural abundance. For such experiments, enrichment of ^{15}N nuclei in the molecules is required and it is performed by chemical syntheses. The study of larger proteins required the development of 2-4D heteronuclear NMR spectroscopy.

As we have already mentioned above, the most basic principle of enzyme catalysis is the ability of enzymes to lower the transition state energy. What it is important in kinetics and thermodynamics in enzyme-catalyzed reactions it has been obtained by monitoring substrate conversion into products; however, much less is known about the kinetics and energetics of conformational changes in the enzyme molecule (Kern et al, 2005). Many enzymatic reactions occur on time scales of microseconds to milliseconds and it has been suggested that dynamics of the enzyme on these time scales is linked to catalysis (Fersht, 1999). Several techniques have been used to detect dynamic processes of enzymes during catalysis, such as fluorescence resonance energy transfer (FRET), atomic force microscopy and stopped flow fluorescence. While those methods report on dynamics of individual sites, or motions of the entire enzyme molecule, NMR spectroscopy of proteins in solution allows detection of motions at a multitude of specific atomic sites simultaneously over a time from picoseconds (Palmer, 1997). These studies suggest that protein motion plays important roles in all aspects of catalysis.

4.2.2 Solid-state NMR spectroscopy

Although progress in protein structure determination has been tremendous over the last years, however large classes of proteins cannot be investigated using liquid state NMR or X-ray crystallography, because either the proteins cannot be crystallized to a sufficient

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diffraction quality for X-ray diffraction, or they cannot be brought into a sufficiently concentrated solution, or are too large for liquid-state NMR (Böckmann, 2006). Therefore there is a considerable interest in the development of methods for protein structure determination, which do not have these limitations. High-resolution solid-state NMR (SSNMR) is a very promising technique in this respect, and is becoming of increasingly importance in studying enzymes whose active sites in complex with substrates exhibit compression, and where key reactive groups have particularly short non-bonded distances and are expected to show unusual dynamics (McDermott & Polenova, 2007). Chemically detailed information inherent to NMR, along with the opportunity in stabilizing species at lower temperatures or even study catalysis in its native biological environment, makes SSNMR an especially powerful method.

4.3 Neutron scattering and X-ray diffraction methods

4.3.1 Diffraction data measurements, collection refinement

In the X-ray diffraction data of crystals are included critical 3D-structural information of the crystallized molecules. Such a data collection for single crystals refers to the process of measuring diffracted intensities and to their standard deviations (noise) (McDermott & Polenova, 2007). The quality of the diffraction data determines the accuracy of the final model. Although for macromolecular crystallography, there are many factors that compromise the data quality, however the key to obtain the highest possible quality of diffraction data lies in the data collection strategy (the wavelength, attenuation, detector-to-crystal distance, exposure time, start angle, scan range and oscillation angle) (Cianci et al., 2008). Macromolecular crystals diffract X-rays poorly as compared to crystals of small molecules, and usually tend to have a much shorter lifetime in the X-ray beam. Next step in a protein crystallography project after the diffraction data collection is the processing of data in order to extract the relative intensities of the diffracted X-ray beam. The methodology and software used for data acquisition and structure solution allow the experimenter to obtain a preliminary structure solution within minutes at the end of the data-collection session (Dauter, 2006). The extracted intensities are then used for the calculation of an electron density map into which a model of the protein is built.

4.3.2 Small angle X-ray diffraction

Small-angle X-ray scattering (SAXS) is a technique that allows the study of the structure and interactions of biological macromolecules in solution. SAXS can be used to probe proteins, nucleic acids, and their complexes under various conditions, without the need of crystallizing the sample and without molecular weight limitations inherent in other methods such as NMR spectroscopy (Das & Doniach, 2006). This progress has been achieved by improving the brightness of synchrotron radiation x-ray sources combined with a wide availability of new computational tools for interpreting SAXS measurements. The basic principle of SAXS is to scatter X-ray photons elastically off molecules in solution and to record the scattering intensity as a function of the scattering angle (Pollack, 2011a). The parameters most frequently extracted from a SAXS profile for a biomolecule in solution, sufficiently diluted to avoid the effects of interparticle interference, are radius of gyration *Rg* and forward scattering intensity *I(0)* which are obtained from the Guinier formula $I(q) \approx I(0) \exp(-q^2 R_g^2/3)$, for small momentum transfer *q* (Lipfert & Doniach 2007). With the

introduction of high-brilliance synchrotron sources, it is now possible to obtain high signal-to-noise SAXS profiles of biomolecule samples with x-ray exposures of milliseconds, or even less, with continuous flow mixing (Pollack, 2011b). The SAXS methodology allows the time resolved characterization of the size and shape of structure ranging from small peptides up to whole viruses by means of beam line setups and appropriate mixers; these structures undergo changes on time scales much faster than one second (Das & Doniach, 2006).

4.4 CD-spectroscopy

4.4.1 Protein folding determination

The rapid characterization of new proteins gets of great importance in the fields of proteomics and structural genomics. Circular Dichroism (CD) relies on the differential absorption of left and right circularly polarized radiation by chromophores which either possess intrinsic chirality or are placed in chiral environments. Proteins possess a number of chromophores which can give rise to CD signals. This spectroscopic technique is widely used in the evaluation of the conformation and stability of proteins under various environmental conditions including temperature, ionic strength, etc. The mechanism of protein folding represents one of the major unsolved problems in molecular biology. Knowledge of protein folding pathways and structural characterization of their states are necessary for a thorough understanding of folding which would have an immediate practical impact, as folding and unfolding participate in the control of a variety of cellular processes (Dobson & Karplus 1999). The task for the experimental scientist is to employ a variety of techniques to gather structural and kinetic data which allow models for folding to be proposed and tested. In view of the rates of the various processes, it is usually necessary to use rapid kinetic methods (stopped or quenched flow) as well as manual mixing methods (dead time typically 10-15 sec) in order to probe as much of the refolding process as possible. Stopped flow methods typically have dead times in the range 1 to 10 msec, and involve dilutions of 11-fold or greater).

4.4.2 Characterization of protein (enzyme) secondary structure and stability upon pH, temperature, denaturizing agents etc

One of the main applications of CD for the study of proteins is the estimation of secondary structure of proteins as their peptide bonds are asymmetric; molecules without symmetry show the phenomenon of circular dichroism. In that case, the chromophores of the polypeptide backbone of proteins are aligned in arrays, and their optical transitions are shifted or split into multiple transitions due to "exciton" interactions resulting different structural elements having characteristic CD spectra as it is shown in figure 13 (Sreerama & Woody, 2004). In addition to the intrinsic CD of the protein backbone, when ligands with chromophores bind to proteins they may develop useful strong extrinsic CD bands; also, the aromatic chromophores of proteins, which have bands in the near ultraviolet region, are often in asymmetric environments and can be used to examine whether mutations change the tertiary structure of proteins (Greenfield, 2006).

4.5 Mass-spectroscopy

4.5.1 Enzyme kinetics: Reaction monitoring, proton transfer

Mass spectrometry has emerged as a valuable tool in biochemistry offering unique insight into biological systems with respect to sensitivity and accuracy. More specifically, mass
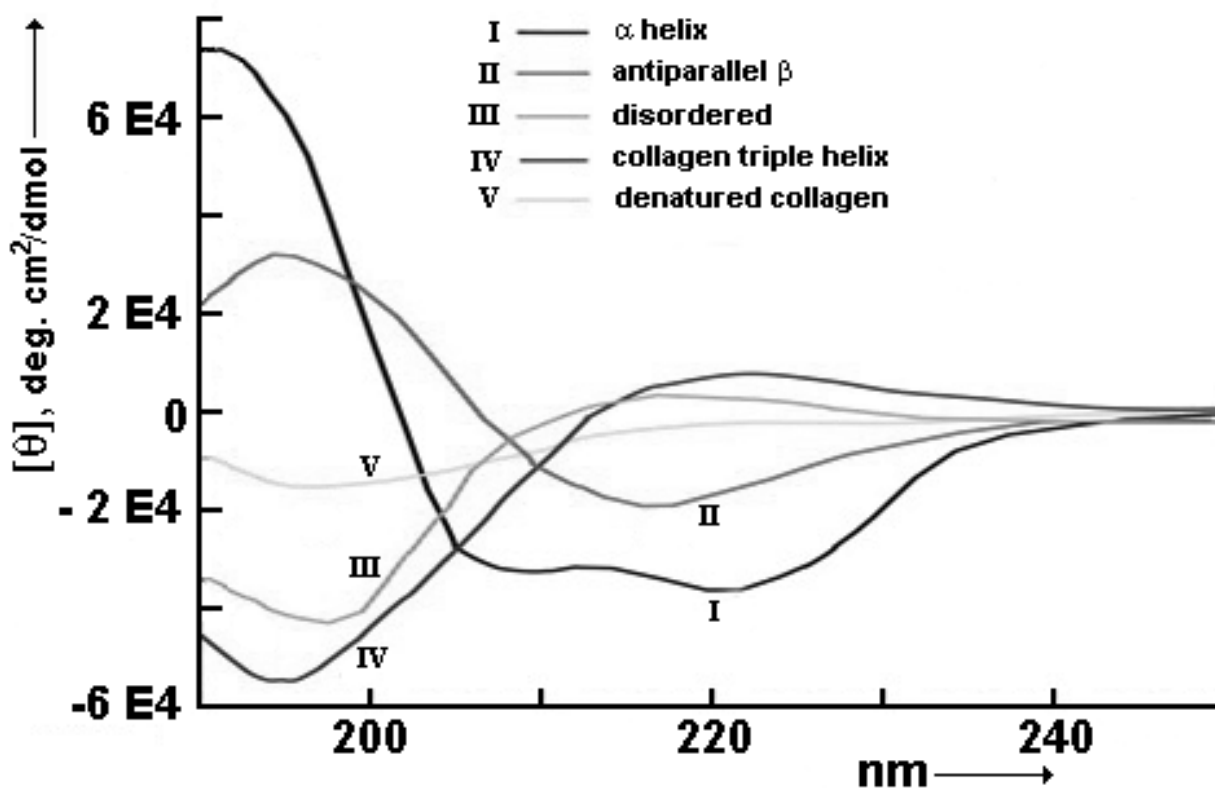
Fig. 13. CD spectra of polypeptides and proteins secondary structures (as modified from Sreerama & Woody, 2004).

spectrometry permits protein sequencing, elucidation of protein folding pathways, characterization of post-translational modifications on peptides and proteins, and detection of covalent and non-covalent protein ligand complexes (Bothner et al, 2000). Recently, the potential of mass spectrometry in studying enzyme catalysis has been widely demonstrated. The development of soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI), has made mass spectrometry an excellent complementary technique to conventional spectrophotometric methods for studying enzyme kinetics. Enzyme mechanisms range from simple two-step processes to complex multi-step reactions and they could be elucidated by means of suitable kinetic experimentation. After the initiation of an enzymatic reaction, there is a short period of time during which reaction intermediates are accumulated; in this "pre-steady-state" period the rate constants of individual steps could be measured. With very few exceptions, pre-steady-state kinetic studies require a time resolution in the millisecond range, which can only be achieved by using automated rapid mixing techniques (Johnson, 1995). Typically, the kinetics in these types of rapid mixing experiments are monitored optically, e.g., by UV-visible absorption or by fluorescence spectroscopy; enzyme kineticists use chromogenic substrate analogues and/or radiolabeled substrates which undergo a change upon turnover. Relatively recently, mass spectrometry (MS)-based techniques have shown great promise in the area of chemical and biochemical kinetics as they do not require chromophoric substrates or radioactive labeling. More important developments of MS spectrometry includes its ability to measure pre-steady-state kinetics of "time-resolved" ESIMS by coupling a continuous-flow-mixing capillary directly to an ESI source (Zechel et al,1998).

Moreover, the kinetics of amide H/D exchange can be measured also by NMR and MS spectroscopy; this latter has been emerged as an attractive alternative to NMR method, with significant contributions to the understanding of the role of protein dynamics in enzyme-catalyzed reactions (Kaltashov & Eyles, 2002).

4.5.2 Protein structure and identification

The determination of protein structure, function and interactions is vital in understanding biological processes. Many biophysical methods have been reported for the investigation of protein structure, dynamics and interactions, in the quest to relate protein structure and function. In the post-genomic era, mass spectrometry (MS) has become the cornerstone of proteomics in identifying cellular proteins and characterizing their expression levels, post-translational modifications, network relationships and metabolism products; they have been developed gentle ionizations techniques for mass spectrometry, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), which expanded remarkably the impact of mass spectrometry in the protein chemistry. The majority of these techniques remain in the area of primary structure determination; however, nowadays mass spectra can provide important clues about higher-order structure of protein by applying several approaches in using MS to study protein higher order structure involving H/D exchange, radical-mediated protein foot-printing and chemical cross-linking with MS (Chance, 2008).

4.5.3 Protein-protein and protein – ligands interactions

As enzyme structure and function are strongly related, the high resolution determination of the molecular structure of proteins gets of great importance in biochemical research. NMR spectroscopy and X-ray crystallography are two important techniques in obtaining high-resolution structural information; however many protein systems are not amenable to these methods due to their size, conformational flexibility, aggregation propensity, or limited sample amount. Therefore, higher throughput methods are necessary to unravel the relationships between protein structural interactions and cellular function, and in this way mass spectrometry (MS) plays an ever-increasing role in protein structure determination due to its speed, sensitivity, and specificity (Heck & van den Heuvel, 2003). The identification and deconvolution of multi-protein complexes helps in understanding protein function and cell regulation, while it is essential to recognize the individual components of protein complexes and their stoichiometries, as well as the nature of interactions, the kinetics and any resulting conformational changes during complex formation. Many protein interactions are non-covalent ones and they can be investigated with MS if the experimental conditions favor retention of non-covalent association (e.g. electro-spray MS, as well as matrix-assisted laser desorption ionization MS) (Heck & van den Heuvel, 2003). More recent applications of mass spectrometry utilize isotopic labeling of proteins via amide hydrogen/deuterium exchange followed by proteolytic fragmentation of labeled protein and analysis; this latter application of MS can measure rates of amide hydrogen exchange with solvent in defined regions of proteins.

4.6 Inorganic mechanisms as probes in investigating enzymatic mechanisms

Sometimes the elucidation of inorganic mechanisms can be used as a probe in investigating particular enzymatic mechanisms which otherwise they may remain at least not clarified or

even unexplored. A good example could be an insight in the mechanism of action of carbonic anhydrase II (EC 4.2.1.1.), a zinc metalloenzyme widespread in nature, which catalyzes the reversible reaction of hydration of CO_2. In living organisms, carbonic anhydrase is involved in various physiological functions including respiration, pH and acid-base regulation, CO_2 and HCO_3^- transport, water and electrolyte balance, and biomineralization; the activity of carbonic anhydrase increases as preceding to the occurrence of the first calcite and quartz crystals formation, in embryos of *Mytilus edulis* L., due to the acceleration of bicarbonate formation. Our example comprises a detailed experimental and theoretical evidence of the effect of carbonic anhydrase II on the dissolution process of carbonate minerals of phosphate ores by dilute acetic acid solutions in the pH range from 2.37 to 6.40. Besides, the elucidation of the mechanism of carbonic anhydrase was based on two relative detailed mechanisms which were reported previously on the selective dissolution of calcite from low-grade phosphate ores. Carbonic anhydrase II was found active at pH values as low as 2.37, even though this enzyme was considered as having a relatively limited pH range of activity at pH 6.0 to 7.8 (Vaimakis & Papamichael, 2002). As a conclusion, two possible mechanisms (figure 14) of action for this enzyme were proposed from pH 2.37 up to 6.40, while the role of carbonic anhydrase in the biomineralization process of marine and other organisms was elucidated.
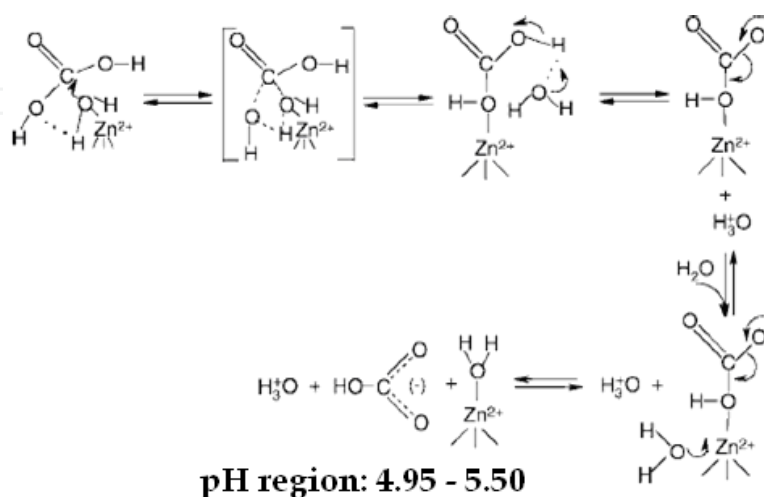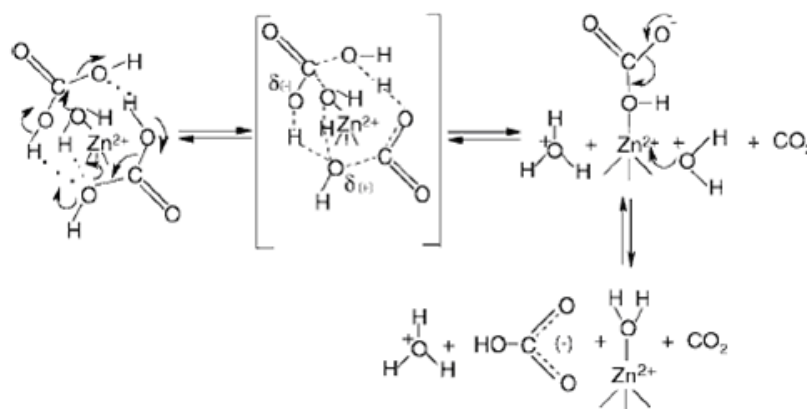

Fig. 14. Two possible mechanisms of action of carbonic anhydrase II (EC 4.2.1.1.), in acid pH-values

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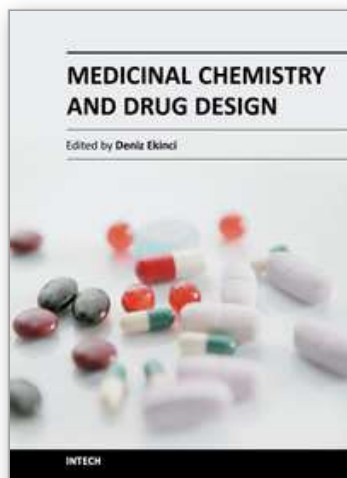
Medicinal Chemistry and Drug Design

Edited by Prof. Deniz Ekinci

Over the recent years, medicinal chemistry has become responsible for explaining interactions of chemical molecules processes such that many scientists in the life sciences from agronomy to medicine are engaged in medicinal research. This book contains an overview focusing on the research area of enzyme inhibitors, molecular aspects of drug metabolism, organic synthesis, prodrug synthesis, in silico studies and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in medicinal chemistry and drug design. Particular emphasis is devoted to both theoretical and experimental aspect of modern drug design. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in associated areas. The textbook is written by international scientists with expertise in chemistry, protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the textbook will enhance the knowledge of scientists in the complexities of some medicinal approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of medicinal chemistry and drug design.

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