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REVIEW ARTICLE

Current problems in mechanistic studies of serine and cysteine proteinases

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Introduction

Serine proteinases are among the most extensively studied enzymes (for recent reviews see Blow, 1976; Kraut, 1977; Huber & Bode, 1978). Their best known representatives, like trypsin and chymotrypsin, are pancreatic enzymes that are related through evolution; serine proteinases also include the bacterial subtilisins, which evolved through a different route, and enzymes that participate in the cascade reactions of blood clotting and complement activation, as well as enzymes that play an important role in phage maturation, fertilization, and in a number of other fields of biological phenomena. Most of the mechanistic studies have been performed with chymotrypsin. Those studies established the basic features of the catalytic action by the 1960's (cf. Bender & Kézdy, 1965). The principal results are summarized in Fig. 1.

It is seen that the nucleophilic attack by the hydroxyl group of the 'reactive' serine residue on the carbonyl carbon atom of the substrate is catalysed by a histidine residue as a general base. This leads to the formation of the tetrahedral intermediate and an imidazolium ion. The intermediate breaks down by general acid catalysis to an acyl-enzyme, an imidazole base, and alcohol or amine. The acyl-enzyme is hydrolysed through the reverse reaction pathway. Of course, in hydrolysis the hydroxyl group of a water molecule is the nucleophile instead of the hydroxyl group of the serine residue. This mechanism implies a close contact between the tetrahedral intermediate and the imidazolium ion, which inhibits the release of proton into the solvent before general acid catalysis (Polgár, 1971), and leads to a one-encounter type reaction (Polgár, 1972).

A new era of mechanistic investigations started with X-ray diffraction studies on chymotrypsin (Matthews *et al.*, 1967; Birktoft & Blow, 1972) and other serine proteinases (cf. Kraut, 1977), which rendered it possible to clothe elementary reaction steps with structural features. Several intriguing questions may be raised in this respect. First of all, as enzyme-specific substrate adducts are not amenable to current X-ray diffraction measurements, what is the reality of the stereochemical mechanisms derived from enzyme–inhibitor or enzyme–substrate analogue complexes? What is the mechanistic role of the serine–histidine–aspartate catalytic triad, which has been a subject of debate over the past decade? What is the contribution to catalysis of the exquisite environment (oxyanion hole) around the negative oxygen atom of the tetrahedral intermediate? How can the established chemistry obtained on small molecules, e.g. stereoelectronic theory and isotope effects, be applied to enzyme catalysis?

Most of the above questions related to the serine enzymes also emerge in the case of cysteine proteinases. The protagonist of these wide-spread enzymes is papain, a plant proteinase (Glazer & Smith, 1971), the only cysteine proteinase until recently whose steric structure was available (Drenth *et al.*, 1971*a*,*b*). Other related thiol enzymes

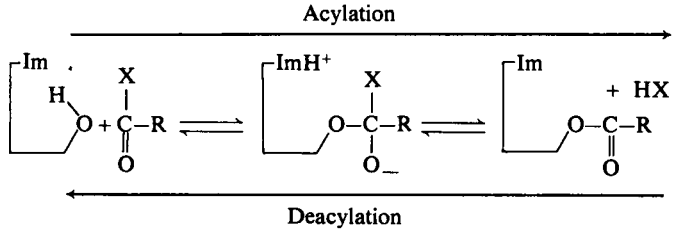

Fig. 1. *Scheme of the reaction mechanism for serine proteinases*
X stands for an OR′ or an NHR′ group in acylation and for an OH group in deacylation.

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of plant origin are, for example, chymopapain, ficin, bromelain (Glazer & Smith, 1971), papaya peptidases (Lynn, 1979; Polgár, 1981 and references therein), asclepains (Brockbank & Lynn, 1979; Lynn *et al.*, 1980) and actinidin. The latter enzyme has recently aroused interest as its three-dimensional structure proved to be remarkably similar to that of papain (Baker, 1977, 1980). Cysteine proteinases are also synthesized during germination of plant seeds (see, for example, Baumgartner & Chrispeels, 1977); they are produced by bacteria, for instance streptococcal proteinase (Liu & Elliott, 1965), and they are found among cathepsins, such as cathepsin B (Keilova & Turkova, 1970).

Cysteine proteinases operate through the formation of an acyl-enzyme intermediate which is a thiol ester, whose formation and decomposition are assisted by a histidine residue (for reviews of mechanistic features see Lowe, 1976; Polgár, 1977; Brocklehurst *et al.*, 1981*a*). There are, however, several uncertainties in the details of the mechanism of action. What are the stereochemical features of the catalysis by papain? Does catalysis take place in a single steric position, as derived from X-ray diffraction studies (Drenth *et al.*, 1976), or it is a two-state mechanism as suggested recently (Angelides & Fink, 1979*b*)? What is the role of the carboxyl group which is situated at or near the active site? Is the tetrahedral intermediate stabilized in an oxyanion hole? Is the thiolate–imidazolium ion-pair the predominant form of native papain?

The growing interest in proteinases has produced many interesting results over the last few years. A complete survey of these data is not practical due to space limitation. Therefore, we concentrated on the issues thought to be most important from the mechanistic point of view, such as the questions addressed above, and tried to analyse critically the often contradictory data.

Serine proteinases

Stereochemistry

The structures of serine proteinases and of their derivatives have been determined by X-ray diffraction measurements at very high resolutions: β-trypsin at 0.15 nm (Bode & Schwager, 1975; Chambers & Stroud, 1979), *Streptomyces griseus* protease A at 0.18 nm (Sielecki *et al.*, 1979) and γ-chymotrypsin at 0.19 nm (Cohen *et al.*, 1981). Unfortunately, the hydrolysis of specific substrates cannot be followed by present-day X-ray diffraction techniques because the catalytic intermediates are transient species of short life-time. In contrast, direct structural information is available for stable enzyme derivatives or complexes formed with inhibitors and substrate analogues which are structurally related to the catalytic intermediates (cf. Kraut, 1977; James, 1980). On the basis of chemical considerations, and assuming that some structural features of the enzyme–inhibitor complexes are shared by the true catalytic intermediates, one can build acceptable models with different geometries. For example, in the case of chymotrypsin the tetrahedral adduct, the key intermediate of the catalytic process, may be located in two positions. In one the tetrahedral adduct exhibits a *syn*-periplanar (Polgár & Asbóth, 1974), in the other an *anti*-periplanar (Bizzozero & Dutler, 1981) arrangement of the carbon atoms C^{β} of serine and C^{α} of the substrate. In the *syn*-periplanar model the imidazole nitrogen can readily approach both the donor and the acceptor atoms of the tetrahedral intermediate within a hydrogen bond distance. By contrast, in the *anti*-periplanar model the imidazole nitrogen is too far from the leaving atom to form a hydrogen bond. Hence, for proton transfer to occur, one must assume that the imidazole moves between the serine oxygen and the leaving atoms as a mobile flip–flop, which process may be associated with structural changes in a relatively large section of the protein (Kraut, 1977; Bizzozero & Dutler, 1981). Such fast movement of the imidazole ring has been regarded to be quite unlikely due to a considerable rigidity of the active site (Komiyama & Bender, 1979). In contrast, when various derivatives of serine proteinases were compared, differences in the positions of the imidazole ring were observed by X-ray diffraction (Brayer *et al.*, 1979; James, 1980).

The position of the tetrahedral adduct in the *anti*-periplanar model is similar to that observed in the trypsin–trypsin inhibitor complex which was thought to be a covalent tetrahedral adduct (Rühlmann *et al.*, 1973; Sweet *et al.*, 1974). On this basis, and because other noncatalytic derivatives of serine proteinases were also found in that position, the *anti*-periplanar geometry has usually been assigned to the catalytic intermediates formed with specific substrates. It may be noted in this respect that in the trypsin–trypsin inhibitor complex the distance between serine oxygen and carbonyl carbon atoms is 0.26 nm (Huber & Bode, 1978), much greater than that required for a covalent bond of the tetrahedral adduct. The 0.26 nm distance would rather be consistent with a van der Waals contact diminished by steric compression. In fact, recent ^{13}C n.m.r. studies of the trypsin–trypsin inhibitor complexes (Baillargeon *et al.*, 1980; Richarz *et al.*, 1980) indicate that they are not true tetrahedral adducts.

It may be mentioned that detection of the tetrahedral intermediate was claimed not only in the trypsin–trypsin inhibitor complex but also in the reaction of anilide substrates with trypsin and elastase (Hunkapiller *et al.*, 1976; Petkov, 1978; Fink & Meehan, 1979; Compton & Fink, 1980). These results were based on observation of 'burst' kinetics obtained with stopped-flow and sub-zero

temperature measurements. However, the possibility of demonstration of a tetrahedral intermediate could not be confirmed (Markley *et al.*, 1981) by re-examination of the data. It was shown that confusing spectral changes can arise from incomplete mixing, thermal gradients or heterogeneity of the substrate. The above failures of demonstrating the tetrahedral intermediate, of course, do not rule out the existence of this species, which was substantiated by serious arguments (Bender & Kézdy, 1965; O'Leary & Kluetz, 1972).

It appears to us from model building that the tetrahedral intermediates formed with different specific substrates may occupy slightly different steric positions, which can affect considerably the relative contributions by the many factors implicated in catalysis, such as proton transfers, various hydrogen bonds, and strain. This is consistent with the comparative studies on activation parameters of the acylation of subtilisin with enantiomeric substrates (Polgár & Fejes, 1979), which suggested that structurally related substrates can be transformed by the enzyme in different conformations. Another example of different enzyme conformers has been implicated by resonance Raman spectroscopy in studies of acyl-chymotrypsin formed with non-specific substrates (MacClement *et al.*, 1981).

The oxyanion hole

The X-ray diffraction studies have shown that the tetrahedral adduct, which is regarded as a transition state-like intermediate, can be stabilized by two hydrogen bonds from the protein to the negative oxygen (Henderson, 1970; Robertus *et al.*, 1972). In accord with these hydrogen bonds, an electrophilic assistance on the carbonyl oxygen was inferred from low Hammett ρ values (Williams, 1970). The oxyanion binding site was proposed to play a crucial role in the stereospecificity of chymotrypsin. Estimation by molecular mechanics of the relative energies of the tetrahedral intermediates which were formed with the enantiomers of acetyltryptophan derivatives indicated that the interaction energy in the oxyanion hole is more favourable with the L-isomer relative to the D-isomer (DeTar, 1981).

Recent experiments also supporting the importance of the oxyanion hole utilized the reactions of thiono substrates, which contain a sulphur atom in place of the carbonyl oxygen (Asbóth & Polgár, 1982). These substrates practically failed to react with chymotrypsin and subtilisin, while their reactivity in alkaline hydrolysis remained similar to that of the corresponding oxygen analogue. With the most specific substrates, decreases in acylation rates were more than four orders of magnitude on substituting sulphur for the oxygen atom. This implies the importance of the oxyanion binding site, into which the sulphur atom cannot fit or with which it cannot form the appropriate hydrogen bonds.

Substrate distortion

On binding of a substrate to the enzyme, some part of the binding energy may be converted into strain energy, which distorts the bond undergoing reaction toward the transition state (Jencks, 1969). The possibility of such a distortion of peptide substrates was also raised in the binding to serine proteinases. Thus the concept of torsional strain implies that binding imposes a *cis*-distortion on a *trans*-peptide bond, which facilitates *cis*-addition of nucleophile and proton to the peptide bond to be cleaved (Mock, 1976).

According to another concept, the catalytic serine reacts with a tetrahedrally distorted and therefore electrophilic carbonyl carbon atom of the substrate, simply because the serine hydroxyl is poised initially in the ideal position to do so (Kraut, 1977; Matthews *et al.*, 1977). Such activation of substrate by strain was proposed on the basis that in crystals of subtilisin the serine oxygen is apparently not activated by hydrogen-bonding to the imidazole nitrogen. Although activation of substrate may indeed contribute to the catalysis, the following problems are associated with this mechanism. (1) Formation of the tetrahedral intermediate is a stepwise reaction rather than a concerted, general base-catalysed process as indicated by kinetic deuterium isotope effects, which implies activation of the serine by the imidazole group (Bender & Kézdy, 1965). (2) Bearing a proton on the serine oxygen, a highly unstable intermediate is formed. (3) Proton transfer from the serine oxygen to the imidazole nitrogen is only possible through a high energy barrier because the two heavy atoms are not located within a hydrogen bond distance. In our opinion, this distance must be reduced either during the formation of the Michaelis complex or at the very beginning of the covalent bond formation, so that general base catalysis be possible.

In support of the substrate distortion mechanisms, the trypsin–trypsin inhibitor complex was again invoked, where the peptide carbonyl carbon atom is indeed tetrahedrally distorted (Huber *et al.*, 1974). The question whether this model is relevant or not to the catalytic intermediate cannot be answered at present, but it should be noted that this particular peptide bond is distorted to some extent even in the free inhibitor (Deisenhofer & Steigemann, 1975). Another important point is that if the torsional strain occurs in acylation, this would oppose stereoelectronic control (Mock, 1976). This is to be discussed next.

Stereoelectronic control

This concept postulates that cleavage of a C–O or

C–N bond formed between C and one of the three heteroatoms of a tetrahedral intermediate is allowed only if each of the other two heteroatoms of this species has a lone-pair orbital oriented *anti*-periplanar to the bond to be broken (Deslongchamps *et al.*, 1975). If stereoelectronic control is applied to the hydrolysis of peptide substrates by chymotrypsin (Bizzozero & Zweifel, 1975; Petkov *et al.*, 1978; Bizzozero & Dutler, 1981; Dugas & Penney, 1981), on the formation of the tetrahedral intermediate, the nonbonded pair of electrons on the leaving nitrogen atom points toward the solvent and its N–H bond toward the inside of the enzyme. Accordingly, protonation of the leaving nitrogen by the histidine, which is necessary for the decomposition of the intermediate, is not possible. Therefore, an inversion at the leaving nitrogen is required to interchange orientation of the N–H bond and the non-bonded orbital (Bizzozero & Dutler, 1981; Dugas & Penney, 1981).

Stereoelectronic theory was elaborated for the reactions of simple organic molecules. However, there are considerable differences between the tetrahedral intermediates formed in enzyme catalysis and in simple organic reactions. Thus, in serine proteinases two electron pairs of the oxyanion of the tetrahedral adduct are hydrogen-bonded in the oxyanion hole and the orientation of the remaining nonbonded pair is defined by these two hydrogen bonds. This restriction was disregarded in the proposals which suggested that one of the three lone pair orbitals would be able to occupy the required position. However, the orientation of the only lone pair which is indeed free in the oxyanion hole appears to be favourable for the decomposition of the tetrahedral intermediate in acylation only (C–N bond cleavage), but not in deacylation ($C–O_G$ bond cleavage) as seen from Fig. 2 (Asbóth & Polgár, 1982). This holds both for the *syn*- and for the *anti*-periplanar model of the tetrahedral adduct discussed above. As for the possibility of inversion on the nitrogen, it is a further question that, if the leaving-group is bound to the protein, inversion could occur as in ammonia, a model compound referred to in support of inversion (Bizzozero & Dutler, 1981).

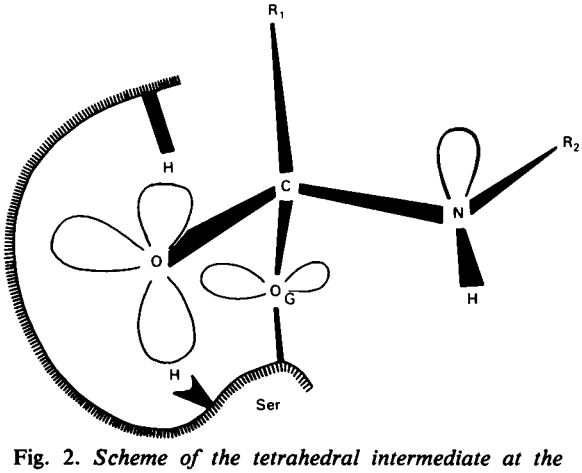

Fig. 2. *Scheme of the tetrahedral intermediate at the active site of serine proteinases*
The free lone pair of the oxyanion is *anti*-periplanar to the C–N bond but not to the $C–O_G$ bond (Asbóth & Polgár, 1982).

The catalytic triad

X-ray diffraction studies on chymotrypsin have shown for the first time that in a serine proteinase the catalytic histidine interacts not only with the active site serine residue but also with an aspartate carboxyl group (Blow *et al.*, 1969). In all serine proteinases examined later by X-ray diffraction the aspartate was found in a similar position (Kraut, 1977). Blow *et al.* (1969) proposed that the triad of aspartate, histidine and serine constitutes a charge relay system that would function by transferring the negative charge from the carboxylate ion through the imidazole to the serine oxygen atom, thereby greatly enhancing the nucleophilicity of this oxygen that attacks the substrate. The concept of charge relay was questioned on chemical grounds (Polgár & Bender, 1969; Polgár, 1972; Rogers & Bruice, 1974). It was suggested that the proton on $N^{\delta 1}$ may be hydrogen bonded but not transferred to the aspartate ion (Polgár & Bender, 1969; Polgár, 1972). ^1H n.m.r. studies (Robillard & Shulman, 1972, 1974*a*,*b*) on the $N^{\delta 1}–H$ bond of the imidazole group of different serine proteinases also suggested only partial proton transfer through this hydrogen bond to the aspartate ion. On the other hand, Hunkapiller *et al.* (1973) claimed to have detected complete proton transfer to the aspartate residue by ^{13}C n.m.r. studies on α-lytic proteinase specifically enriched with [2-^{13}C]histidine. Although this conclusion was drawn from n.m.r. data close to the noise level (Egan *et al.*, 1976), it was widely accepted in the literature as a proof in favour of the charge relay system. Other data, like those obtained with difference infrared titration (Koeppe & Stroud, 1976) were also interpreted at that time in terms of an effective charge relay (for a review see Kraut, 1977).

Probably the most powerful and direct n.m.r. technique for probing the charge relay utilized ^{15}N n.m.r. (Bachovchin & Roberts, 1978). This study on α-lytic proteinase specifically enriched with [N^{τ}-^{15}N]histidine and [N^{π}-^{15}N]histidine demonstrated that, on protonation of the triad, the proton stayed on the histidine rather than on the aspartate residue. ^1H n.m.r. studies on the C2–H of histidine in trypsin

were also inconsistent with the charge relay mechanism (Markley & Ibanez, 1978).

A comparative ^1H n.m.r. study on subtilisin and thiolsubtilisin provided information about the role of the triad during the catalytic process in its ground and transition states, respectively (Jordan & Polgár, 1981). Thiolsubtilisin is obtained by chemical modification of the serine enzyme and differs only in having an –SH group in place of the catalytic –OH group (Polgár & Bender, 1966; Neet & Koshland, 1966). The cysteine obtained in this way forms a mercaptide–imidazolium ion-pair with the neighbouring histidine residue (Polgár, 1974*a*). This charge distribution resembles the ion-pair formed during catalysis by the parent serine enzyme, i.e. charge distribution of the negatively charged tetrahedral adduct and the protonated histidine. The very low field ^1H n.m.r. resonance that is characteristic of the hydrogen bond between the imidazolium and aspartate of the catalytic triad was not found in native subtilisins (type Novo or Carlsberg) but was present in thiolsubtilisins and in the phenylboronic acid derivatives of the serine enzymes (Jordan & Polgár, 1981). The latter derivative can also be regarded as a transition state analogue bearing a negative charge (Matthews *et al.*, 1975). These results may indicate that it is at the tetrahedral transition state that the hydrogen bond between the protonated histidine and the aspartate residue is more important than at the other stages of the catalysis. This is consistent with previous suggestion (Polgár & Bender, 1969).

Neutron diffraction, which can locate hydrogen atoms experimentally, is another important method to examine the issue of charge relay. This technique was employed on crystalline trypsin covalently inhibited with a transition state analogue, the monoisopropylphosphoryl group (Kossiakoff & Spencer, 1980, 1981). The results clearly indicated that the histidine, rather than the aspartate, was the recipient of the proton.

Proton inventory based on rate measurements in mixtures of ^2H_2O and ^1H_2O is a helpful means to estimate the number of protons involved in catalysis by serine proteinases (Kresge, 1973; Schowen, 1978; Schowen & Schowen, 1982). If the charge relay system is at work, two protons are expected to move simultaneously: one from serine to histidine, and the other from histidine to aspartate. If the charge relay does not function, there is only one proton transfer, and thus the aspartate remains unprotonated. The measurements indicated that hydrolysis of oligopeptides produced a proton inventory consistent with two-proton catalysis, whilst simple substrates exhibited a one-proton mechanism (Pollock *et al.*, 1973; Hunkapiller *et al.*, 1976; Elrod *et al.*, 1980). These data might support the charge relay mechanism at least for extended substrates and they are not inconsistent with this mechanism even for simple substrates provided that the two proton transfers occur consecutively (Hunkapiller *et al.*, 1976). Furthermore, one may argue that solvent isotope effects are more informative diagnostics of proton participation in transition state than are n.m.r. or neutron diffraction measurements, which analyse stable enzyme forms. However, because in the substrate-free enzyme protonation of the imidazole group does not lead to protonation of the aspartate ion, proton transfer would be even less probable during the catalytic action, since a considerable electrostatic effect arising from the negatively charged tetrahedral intermediate counteracts this process (see the following discussion of the quantum chemical results). Although the two-proton catalysis observed in proton inventory studies is consistent with the charge relay mechanism, it should be kept in mind that the identity of proton donors and acceptors cannot be established with this kinetic method, and the data with the more complex substrates may also reflect secondary isotope effects, conformational change, or some other phenomenon, which complicates interpretation of the data (Kresge, 1973; Elrod *et al.*, 1980; Schowen, 1978). Nevertheless, the above results clearly indicate that with different substrates somewhat different catalytic machineries of the enzyme are called into action (Elrod *et al.*, 1980).

Molecular orbital studies were also undertaken to disperse the cloud surrounding the catalytic triad (see for a review Náray-Szabó & Bleha, 1982). Most of the earlier calculations supported the charge relay mechanism, whereas recent studies have led to the opposite conclusion. The important corollary of the recent calculations is that the negative tetrahedral intermediate should stabilize the imidazolium–aspartate ion-pair relative to the imidazole–aspartic acid form, i.e. formation of the tetrahedral adduct acts against the charge relay. Conversely, the negative aspartate ion stabilizes the ion-pair form of the imidazole–tetrahedral intermediate couple (Umeyama *et al.*, 1981; Kollman & Hayes, 1981; Náray-Szabó *et al.*, 1982). Such an electrostatic catalytic role, as opposed to being the ultimate proton acceptor of a charge relay system, was earlier proposed for the aspartate ion (Polgár, 1972). The addition of aspartate ion to the negatively charged tetrahedral adduct and the imidazolium ion produces a symmetrical charge distribution (− + −), which is stabilized by the extended hydrogen bonding system formed in the transition state of the catalysis, as shown in Fig. 3 (Polgár, 1972). It was pointed out that ionic triads, such as − + −, are indeed considerably stabilized by polarization effects (Warshel, 1978).

Summarizing, we may conclude that the possible advantage of the charge relay mechanism is not

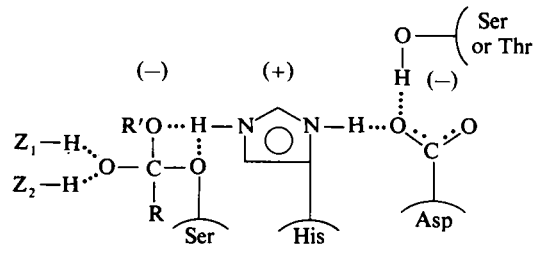

Fig. 3. *Scheme of the extended hydrogen-bonding system of serine proteinases involving the oxyanion hole, the substrate and the catalytic triad (Polgár, 1972)*

clear, because a gain in the general base-catalysed step would be lost in the subsequent general acid-catalysed step (Polgár, 1972). Were the charge relay operative, a critical catalytic factor, transition state stabilization by the aspartate ion, would be sacrificed.

Cysteine proteinases

Stereochemistry of papain catalysis

The three-dimensional structure of papain was determined by X-ray diffraction measurements (Drenth *et al.*, 1968). On the surface of the molecule there is a large groove wherein the essential thiol group of cysteine-25 is situated next to the imidazole ring of histidine-159. The binding mode of the substrate in the groove was derived from the difference-Fourier maps obtained with chloromethylketone substrate analogues covalently attached to the sulphur atom (Drenth *et al.*, 1976). By removing the methylene group, which is between the carbonyl carbon and the sulphur atoms, it was possible to construct the models of the acyl-enzyme and the tetrahedral intermediate. Similarly to that found with serine proteinases, the oxyanion of the tetrahedral adduct of papain also seems to be stabilized by two hydrogen bonds: namely, from the backbone –NH– group of cysteine-25 and from the $-NH_2$ group of glutamine-19. This is the main difference compared with the stereochemical mechanisms suggested previously (Wolthers *et al.*, 1970; Lowe & Yuthavong, 1971) where the oxyanion was proposed to exist in a sterically unrestricted environment. This earlier mechanism is supported by the kinetics of acylation of papain with methyl hippurate and the corresponding thiono derivative, which show similar rate constants for the two substrates (Lowe & Williams, 1965; Asbóth & Polgár, 1982), in sharp contrast to what is observed with serine proteinases (see the section on the oxyanion hole). It should be emphasized, however, that the most specific peptide substrates may indeed require the oxyanion binding site. Another feature of papain catalysis, which is related to the mechanism of action of serine proteinases, may be a 30° rotation of the imidazole ring between the sulphur atom and the leaving group (Drenth *et al.*, 1976).

A conformational change was also suggested to occur during catalysis by papain (Angelides & Fink, 1978, 1979*a*,*b*). The key feature of the mechanism is the existence of two conformational states of the enzyme, one with the imidazole of histidine-159 hydrogen bonded to asparagine-175 (catalytically inactive, 'up' position) as found by X-ray diffraction measurements, and the other involving the imidazole protonated and electrostatically interacting with the carboxylate of aspartate-158 and the thiolate of cysteine-25 (catalytically active, 'down' position). The substrate can only bind to the sterically active 'up' conformation, and during the catalytic action it is moving between the two positions. There are, however, several difficulties in this two-state mechanism. (1) There is no evidence that would support the inactivity of the 'up' position. Moreover, X-ray diffraction studies clearly indicate that the imidazole of His-159 is in an ideal position to interact with and thus activate the thiol group of cysteine-25 (Drenth *et al.*, 1976). (2) The thiolester intermediate exhibits an *anti*-periplanar conformation in the 'up' position, whereas it is *syn*-clinal in the 'down' position. Such a rearrangement of the acyl group in the limited space available in the active site groove, in which the substrate is fixed by a number of hydrogen bonds, appears to be quite unlikely without breaking some of these bonds (B. Asbóth & L. Polgár, unpublished work). (3) According to the proposed mechanism (Angelides & Fink, 1979*b*), the 'down' position should be the predominant form of papain, and the 'up' position is found only in the inactive crystals grown at high pH (9.3). However, the crystallographic studies of actinidin at 0.17 nm resolution and pH 6 (Baker, 1980) where the analogous 'down' conformation, if it indeed exists, is expected to be seen, have shown that the enzyme structure is closely similar to the 'up' conformation of papain. Nonetheless, a possible 'down' position may be active toward some reactants, like *n*-propyl 2-pyridyl disulphide (Brocklehurst *et al.*, 1981*b*). It is worth noting in this regard that the existence of at least two reactive conformers of papain was deduced from alkylation reactions of cysteine-25, but no assignment to definite structures was made (Polgár & Halász, 1978). Conformational change of the active site was also inferred from the reaction of papain with mercuric ion (Sluyterman *et al.*, 1977) as well as from kinetic measurements with a 2-pyridyl disulphide derivative (Brocklehurst *et al.*, 1979).

Role of aspartate-158 in catalysis

In the early studies on papain action, it was thought that a carboxyl group rather than an imidazole functioned as a general base (Bender & Brubacher, 1966; Kirsch & Igelström, 1966; Drenth *et al.*, 1971*a*; Löffler & Schneider, 1974). The validity of this assumption was questioned (Husain & Lowe, 1968) and was practically ruled out by the determination of the steric structure of the enzyme, which showed that the nearest carboxyl group, which belongs to aspartate-158, was at a distance of 0.75nm from the sulphur atom of cysteine-25 (Drenth *et al.*, 1970, 1976). Based on the magnitude of the Hammett ρ value for the rates of deacylation of substituted benzoyl-papains, Zannis & Kirsch (1978) claimed that it was most likely that the deacylation of nonspecific acyl-enzymes is catalysed by a carboxylate group as a general base. Referring to these data, Angelides & Fink (1979*b*) raised the possibility that deacylation of specific substrates could also be assisted by aspartate-158. The difficulties in the interpretation of ρ values in enzyme reactions were discussed in detail by Johnson *et al.* (1981*b*). They concluded that the direct involvement of an uncharged histidine residue in the deacylation step in papain-catalysed reactions cannot be excluded. Johnson *et al.* (1981*b*) have also pointed out that the near-zero heat of ionization of the catalytic group of $pK_a = 4$, another argument in favour of carboxylate participation (Zannis & Kirsch, 1978), is not inconsistent with the action of imidazole as a general base.

Whereas the direct involvement of aspartate-158 in catalysis by papain is far from being proved, its effects on the ionization of the catalytic groups (cysteine-25 and histidine-159) were demonstrated in several cases. (1) The pH–rate profile of papain reactions is modulated by two acid ionizations rather than a single ionization (Sluyterman & Wijdenes, 1973; Lewis *et al.*, 1978). (2) ^{19}F n.m.r. and fluorescence studies of alkyl and alkylthio derivatives of cysteine-25 also indicate two ionizable groups (histidine-159 and aspartate-158) in the vicinity of cysteine-25 (Bendall & Lowe, 1976*a*,*b*). The ionizations of the two groups are linked, i.e. ionization of one group affects ionization of the other. (3) The effect of aspartate-158 is also apparent in the reaction of 2-pyridyl disulphide probes with papain (Shipton & Brocklehurst, 1978; Brocklehurst *et al.*, 1979). As the contribution by the carboxylate ion is not seen in the reaction with either ficin (Brocklehurst & Malthouse, 1980) or actinidin (Brocklehurst *et al.*, 1981*b*), papain is unique in this respect.

The cysteine–histidine couple

Perhaps the most significant mechanistic difference between catalyses by serine and thiol proteinases is that with serine enzymes the nucleophilic attack on the carbonyl carbon atom of the substrate is assisted by general base catalysis, whereas with thiol enzymes there is no such facilitation. This difference arises from the formation of a mercaptide–imidazolium ion-pair in the free thiol enzyme, which implies that the proton is already on the imidazole when the mercaptide ion attacks the substrate. The arguments supporting ion-pair formation have previously been reviewed (Polgár, 1977). The two most important evidences were the spectroscopic detection of a mercaptide ion-like form of cysteine-25 (Polgár, 1974*b*), and the fluorometric detection of protonated imidazole (Sluyterman & de Graaf, 1970; Lowe & Whitworth, 1974; Sluyterman & Wijdenes, 1976). Zannis & Kirsch (1978) have not accepted the concept of ion-pair formation, and claimed that there was no proof for the participation of histidine-159 in fluorescence quenching. They suggested aspartate-158 as an alternative candidate. It was, therefore, of considerable interest to examine the ionization behaviour of histidine-159 by another method, the more direct proton n.m.r. technique (Johnson *et al.*, 1981*a*; Lewis *et al.*, 1981). These studies offered compelling evidence that histidine-159 is in the protonated form below pH8, where the ion-pair was proposed to exist. The protonation of the imidazole was also confirmed by n.m.r. measurements in the case of thiolsubtilisin (Jordan & Polgár, 1981) that has a similar mercaptide–imidazolium ion-pair (Polgár, 1974*a*). Ion-pair formation in papain is also supported by other recent data, such as potentiometric difference titration (Lewis *et al.*, 1976), alkylations with negatively charged reactants (Halász & Polgár, 1977), and kinetic deuterium isotope effects (Polgár, 1979; Creighton & Schamp, 1980; Creighton *et al.*, 1980; Frankfater & Kuppy, 1981). A closely similar ion-pair is also present in ficin as indicated by alkylations with chloroacetate (Brocklehurst *et al.*, 1982).

A further question that has been raised about the ion-pair of papain concerns the equilibrium between the neutral (thiol–imidazole) and the ion-pair (thiolate–imidazolium) forms of the cysteine–histidine couple. Spectrophotometric difference titrations indicated at least 50% (Polgár, 1974*b*), potentiometric difference titrations 90% (Lewis *et al.*, 1976), solvent deuterium isotope effects 66% (Creighton *et al.*, 1980) and n.m.r. measurements 100% (Johnson *et al.*, 1981*a*), ion-pair form. Although one probably cannot distinguish between a 90% or 100% ion-pair content due to the error in determination, one can conclude from the most direct n.m.r. measurements and from the other supporting data that the ion-pair is the prevailing form of the catalytically active free papain.

Conclusion

A survey of the literature of the last few years brought up many contradictory data about serine and thiol proteinases, perhaps more than one would have desired. The story of the charge relay mechanism is the most striking. Although its weakness had been pointed out, this intriguing hypothesis attracted many followers. When it was most popular, the majority of results happened to be consistent with it; now most results appear to be at variance with it. Another issue, the demonstration of the tetrahedral intermediate by both X-ray diffraction and spectrophotometric measurements, which was frequently referred to as a fact in the past, is under serious criticism at present. A further source of controversy has been the inappropriate application to enzyme catalysis of diagnostic probes elaborated for the reactions of simple molecules. Of course, provided that the diverse effects of the protein environment can be, and are, taken into account, valuable information about enzyme–substrate reactions may be deduced from Hammett ρ values, fractionation factors, stereoelectronic theory, kinetic deuterium isotope studies including protein inventory, and quantum chemical calculations.

A considerable advance is noticeable in the field of stereochemistry, proton movements, transition state stabilization, just to mention a few of the underlying issues of the catalytic mechanism. Thus, it appears that in serine proteinases there is no charge relay mechanism and the oxyanion hole is an essential part of the catalytic entity. In papain catalysis, the oxyanion hole may not be important, at least with common substrates. Some other consequences of the studies in this field are as follows. The thiolate–imidazolium ion-pair as the reactive nucleophile of thiol proteinases has been confirmed. Aspartate-158 may not be directly implicated in the catalysis by thiol enzymes. The two-state mechanism for papain catalysis seems to be an attractive idea but probably not in its form proposed originally. Substrate distortion in the catalysis by serine proteinases is a feature of the mechanism too subtle to evaluate at the present. Although the approximate stereochemistry of the catalysis by serine and thiol proteinases has been revealed, we cannot yet assign precise geometry to any of the true catalytic intermediates. The solution of this central problem must await the application of more sophisticated methods, such as sub-zero temperature X-ray crystallography (Alber *et al.*, 1976).

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