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CRYSTALLOGRAPHIC AND NMR STUDIES OF THE SERINE PROTEASES

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Since the serine proteases form the most thoroughly understood family of enzymes, it is of some interest to consider what the two principal physical techniques of studying enzymes, crystallography and nuclear magnetic resonance (NMR), have told us about the details of their enzymatic mechanism. Due to limitations of space, this review cannot be comprehensive nor can it describe the history of the current ideas concerning the action of these enzymes.

Two major issues have attracted the attention of both diffraction and NMR studies in the past ten years: the first concerns the nature and the role of the catalytic triad of serine 195, histidine 57, and aspartic acid 102; the second is the possibility of induced distortions upon complexation of either the substrate or the enzyme. We first consider these issues in terms of the crystallographic studies and then of the NMR experiments. Finally, we interpret these results in terms of a detailed structural and electronic mechanism. Our conclusions, subject to further test, are that the triad does not exist in the resting state of the enzyme because the serine-histidine bond is not formed. However, we suggest that this bond does form upon complexation with substrates allowing the triad to play an important role in catalysis.

CRYSTALLOGRAPHIC STUDIES OF SERINE PROTEASE STRUCTURE AND MECHANISM

The currently accepted chemical steps in the mechanism of the serine proteases are shown in Figure 1. Crystallographic studies have been

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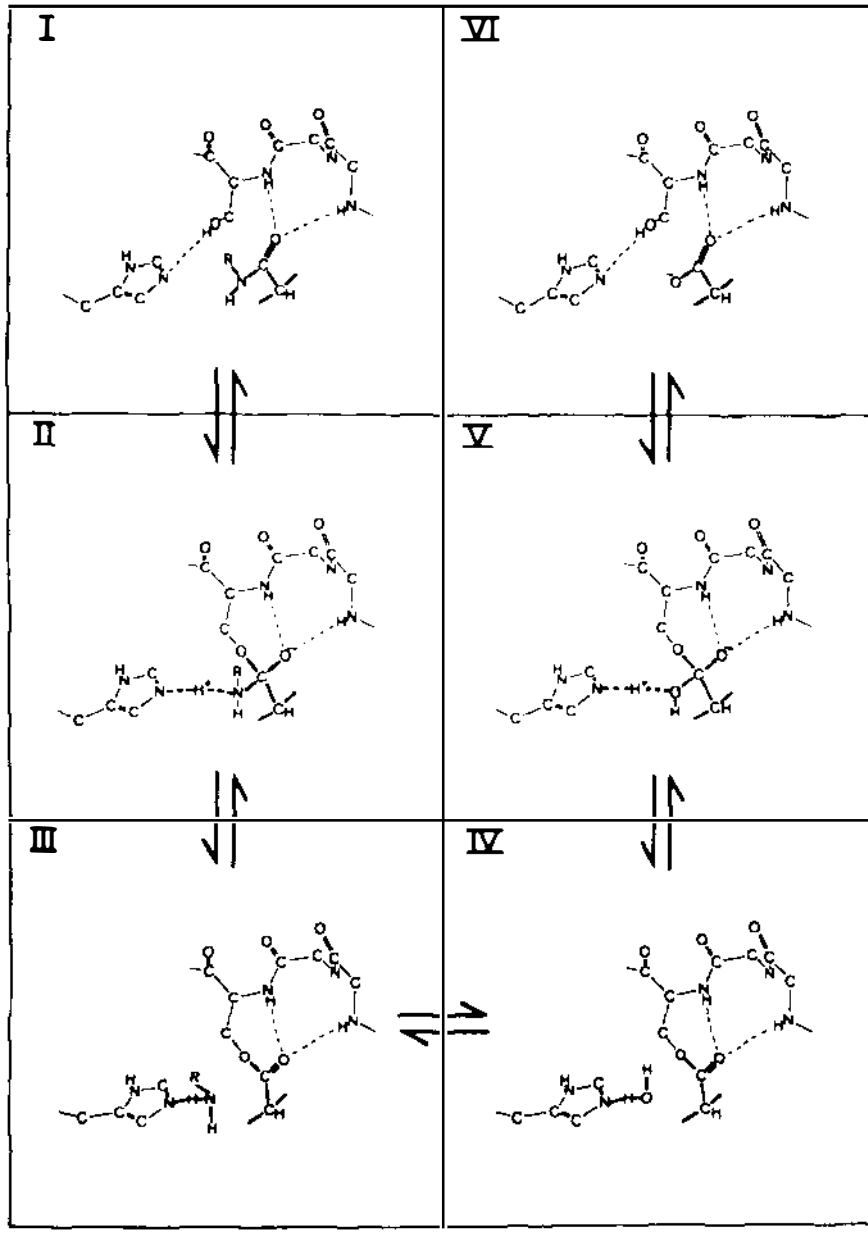


Figure 1 Generally agreed upon sequence of stereochemical steps in the hydrolysis of an amide bond by serine proteases (from Reference 20). *I*. Michaelis complex. We suggest here that the serine proton may be on the His rather than Ser in this pretransition state complex. *II*, *V*. Tetrahedral intermediates. *III*, *IV*. Acyl intermediates. *VI*. Product complex.

completed on several serine proteases in the absence of substrates, on complexes with substrates, and on complexes with substrate analogs that correspond to intermediates along the reaction pathway. We discuss the results of these detailed structural studies by considering each step in the reaction pathway separately.

When Blow and co-workers (9) first observed buried aspartic acid 102 in chymotrypsin and in homologous serine proteases, they suggested that its role in catalysis might be to increase the nucleophilicity of serine 194 by effectively shifting the equilibrium positions of the histidine and serine protons in the direction of the aspartic acid and thereby increasing the negative charge on the serine hydroxyl. The positions of these protons during the catalytic cycle have been a major subject of debate, so that one of the most satisfying results of this survey is to find considerable agreement on different structures, as is discussed below.

Another possible catalytic role of the enzyme in which it induced geometric and electronic strain of the substrate had been proposed and received support from the fact that the structure of the enzyme binding site is complementary to the structure of the tetrahedral intermediate (20, 41, 43). Furthermore, there was a lack of exact complementarity in chymotrypsinogen (7, 56) and trypsinogen (16, 30), and some apparent distortion had been reported in the refined trypsin-trypsin inhibitor complex (22, 23, 53). However, recent NMR experiments have shown that the extent to which the scissile carbon of the Michaelis complex is distorted away from planar in the pretransitional state complex is very small (5, 42). Another possibility is that binding to a potential substrate changes the catalytic triad of the enzyme towards a more reactive state rather than the enzyme changing the substrate. This is seen, in light of the available NMR and crystallographic results, to be quite possible and is discussed below.

Table 1 lists the pancreatic and bacterial serine protease structures that have been solved to date, along with information on the state of their structural analysis. Many of the serine protease structures have been refined at higher than 2 Å resolution, so that the expected error in the coordinates is in many cases 0.2 Å or less. Thus, very definite statements can be made concerning such issues as whether or not hydrogen bonds are formed.

Native Enzyme Structure

A significant result from high resolution refinement of the serine protease coordinates is that no strong hydrogen bond is formed between the γ hydroxyl of serine 195 and the N_3 of histidine 57 in the resting state of the enzyme (11, 12, 15, 33, 37, 50). In both the mammalian and bacterial

Table 1 Independently determined serine protease structures

Enzyme	Resolution (Å)	R-factor[a]	pH	Reference
Mammalian				
tosyl α-Chymotrypsin	2.0	MIR[b]	4.2	25
α-Chymotrypsin	2.8	MIR	4.0	53a
γ-Chymotrypsin	1.9	0.18	5.5	18
Benzamidine-trypsin	1.8	0.23	7.0	13
DIP-trypsin	1.5	0.16	7.5	26
Trypsin-PTI	1.9	0.23	7.0	9
Trypsin-STI	2.6	MIR, $\langle m \rangle = 0.67$	7.0	12,20
Chymotrypsinogen	2.5	0.43		6
Trypsinogen	1.8	0.23	7.0	7
	1.9	0.31	7.5	8
Elastase	2.5	MIR, $\langle m \rangle = 0.86$	5.0	49a
Bacterial				
subtilisin	2.0	0.23[c]	5.9	14
			7.5	
SGPA[d]	1.8	0.14	4.1	16
SGPB[d]	2.8	MIR	4.2	15a
α-lytic protease	2.8	MIR, $\langle m \rangle = 0.83$	7.2	17

[a] R-factor $= \Sigma|F_o - F_c|/\Sigma F_o$, where F_o and F_c are the observed and calculated structure factor amplitudes and the summations are overall reflections.
[b] MIR = multiple isomorphous replacement phased electron density map used to build model. No extensive refinement reported. $\langle m \rangle$ = mean figure of merit.
[c] Unconstrained refinement.
[d] SGPA, B = *Streptomyces griseus* serine protease A and B.

proteases the nitrogen-oxygen distances, which vary between 3.2 and 3.7 Å, are too long for a hydrogen bond. More importantly, the oxygen is at the wrong angle to the histidine nitrogen for optimal hydrogen bond formation. In all cases, extensive and specific water structure is found in the active site region. In particular, there is either a water or a sulfate molecule bound to the serine hydroxyl and a water molecule hydrogen bonded to the N_3 nitrogen of histidine. Two water molecules are found in about the same position in all of the serine proteases. Thus, in the absence of substrates it is apparently true that the catalytic triad is incomplete and the buried aspartic acid 102 does not affect the polarity of serine 195, since there is no direct hydrogen bond between the serine and the histidine.

The absence of a strong hydrogen bond between serine and histidine in the native enzyme has prompted Kraut and co-workers (33, 37) to suggest that both the charge relay mechanism and, by implication, general base catalysis by histidine 57 are not important during catalysis by serine proteases. Rather, the Asp-His couple functions as a "proton

shuttle." However, this conclusion based on the enzyme structure in the absence of substrates is not generally warranted since, as discussed below, a Ser-His hydrogen bond is observed in the structure of trypsin–pancreatic trypsin inhibitor (PTI) (21–23).

Structure of the Pretransition State Michaelis Complex

Good analogs of a pretransition state Michaelis complex of the serine proteases are the trypsin-trypsin inhibitor complexes (Figure 2). High resolution crystal structures of two complexes have been determined: (*a*) the complex between bovine pancreatic trypsin inhibitor (PTI) and trypsin (22, 23, 49), and (*b*) the complex formed by trypsin with soybean trypsin inhibitor (STI) (27, 53). The structure of the PTI complex with trypsin has been refined at 1.9 Å resolution to a crystallographic R-factor of 0.23 (9), so that the coordinates from this structure are somewhat more reliable. Several mechanistically important conclusions can be derived from this crystal structure. First, Huber and colleagues (21, 23) have found that the distance between the serine hydroxyl and histidine nitrogen is 2.7 Å and the geometry is optimal for the formation of an excellent hydrogen bond. Thus in contrast to the resting enzyme, there does seem to be a hydrogen bond between His 57 and Ser 195. Second, with the binding of the inhibitor all of the water molecules in the active site have been removed. Presumably, it is this circumstance that results in the proper hydrogen bonds between the side chains of the catalytic triad. Therefore, some influence of the buried aspartic acid on serine 195 is not excluded in the Michaelis complex. Finally, the carbonyl carbon of the scissile bond of the inhibitor is suitably placed for a nucleophilic attack by serine 195 while the amide nitrogen of the scissile bond is also suitably placed to receive the proton between the serine hydroxyl and the histidine nitrogen, assuming a relatively small movement of His 57. Huber and co-workers (21, 23) find that the distance between the Ser 195 Oγ and the carbonyl carbon of the scissile bond of the inhibitor (Lys-15C) is 2.6 Å, somewhat shorter than van der Waals contact but longer than expected in a covalent tetrahedral compound. The refined structure exhibits a significant distortion of the carbonyl carbon of Lys 15 towards tetrahedral, though not as much as suggested in the unrefined structure. However, conclusive structural evidence is difficult to obtain at 1.9 Å on this issue, given the small changes in coordinates required to go from a trigonal to a tetrahedral carbon arrangement. James et al (25) concluded that the observed Ser Oγ to carbonyl C distance of 2.6 Å implies a deviation of 0.1 Å, of the carbon atom from the plane of the 3 atoms to which it is bonded, using the

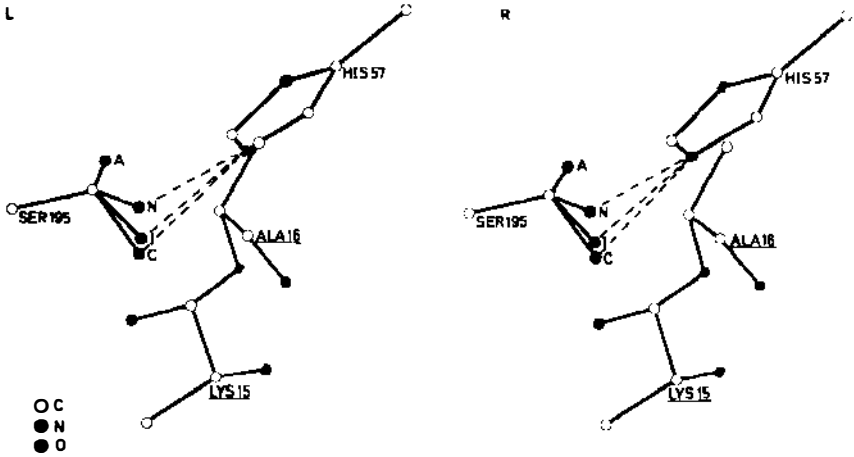


Figure 2 A stereo drawing of His 57 and Ser 195 of trypsin and Ala 16 and Lys 15 of PTI as observed in the crystalline complex (from Reference 21). Also shown are the various Ser 195 Oγ conformations observed in "native" α-chymotrypsin, *A*; tosyl chymotrypsin, *C*; trypsin-PTI, *I*; tentative Oγ position of native trypsin, *N*.

relationship of Burgi et al. (13). As discussed below, NMR studies of the inhibitor complex strongly favor a trigonal carbon.

Tetrahedral Intermediate

The substrate analogs that have been used as models of tetrahedral intermediates in the reaction pathway are the diisopropylphosphoryl (DIP) enzyme (6, 8, 14, 51) and the boronyl complexes (36) with the enzyme. Stroud & Chambers (14) observed in their high resolution refined map of DIP-trypsin that in fact one of the isopropyl groups has been hydrolyzed specifically by the enzyme so that the resulting monoisopropylphosphoryl (MIP) trypsin provides an even better analog of the expected tetrahedral intermediate. In this structure, as in a boronyl complex with subtilisin (36), one oxygen of the tetrahedral adduct is hydrogen bonded to the backbone amides of glycine 193 and serine 195, the so-called oxy-anion hole. The other unesterified oxygen is hydrogen bonded to the histidine N_3.

A most interesting and revealing structural study of the MIP-trypsin complex has been done by Kossiakoff & Spencer using neutron diffraction (31, 32). With neutron crystallography, it is possible to locate with great precision the position of protons or deuterons bound to the protein. Since they scatter neutrons as strongly as the atoms to which they are attached, it was observed (Figure 3) that the histidine 57 has bound a proton on each of its two ring nitrogens at the acidic pH in which the

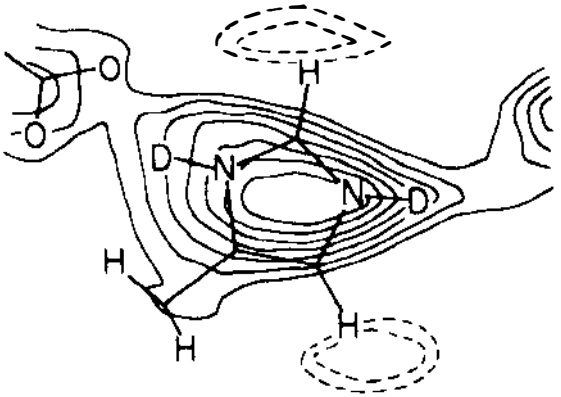


Figure 3 Neutron diffraction map of trypsin active site of monoisopropylphosphoryl trypsin (from Reference 31). This shows that at acid pH, both imidazole nitrogens have a firmly bound proton (deuteron). That is, the proton between His 57 and Asp 102 is bound to His 57. The nomenclature for imidazole atoms used in this review is N_1 and N_3 for $N^{\delta 1}$ and $N^{\epsilon 2}$ respectively.

neutron diffraction studies were done. That is, the proton between histidine 57 and asparatic acid 102 is not associated with aspartic acid's carboxylate, as some studies (24, 29) have postulated, but rather is firmly bound to the histidine nitrogen. This result shows that whatever the role of Asp 102, it does not result in the transfer of a proton from histidine to the carboxylate group. The N_3 proton is also on the histidine and is hydrogen bonded to the oxygen of the phosphoryl group.

Acyl Intermediate

The only good analog of the acyl intermediate that has been examined at sufficiently high resolution to make the structural analysis secure is indoleacryloyl chymotrypsin studied by Henderson (19). At pH 4, indoleacryloyl chymotrypsin is stable for the day required for crystallographic study. Henderson observed that the indole moiety is bound in the pocket of chymotrypsin, as had been found earlier for the indole side chain of formyl tryptophan (52), and that the carboxyl was attached covalently to the serine hydroxyl (Figure 4). However, the carbonyl oxygen of indoleacryloyl is hydrogen bonded via a water molecule to the N_3 of His 57. This is an abortive complex because it is not deacylated at the rate typical of a good substrate. Henderson suggested that the slow deacylation rate results from the observed water molecule that bridges the acyl and imidazole moieties. This water molecule is not in the correct position to deacylate the carbonyl carbon. Henderson also suggested that a natural acyl intermediate (Figure 5) has an imido group preceding the

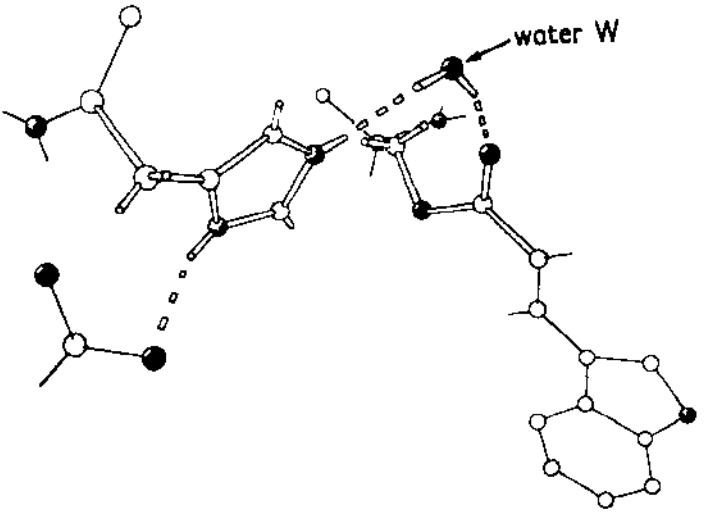


Figure 4 Model of indole acyloyl chymotrypsin showing water molecule between His 57 and the acylating carbonyl oxygen (from Reference 19).

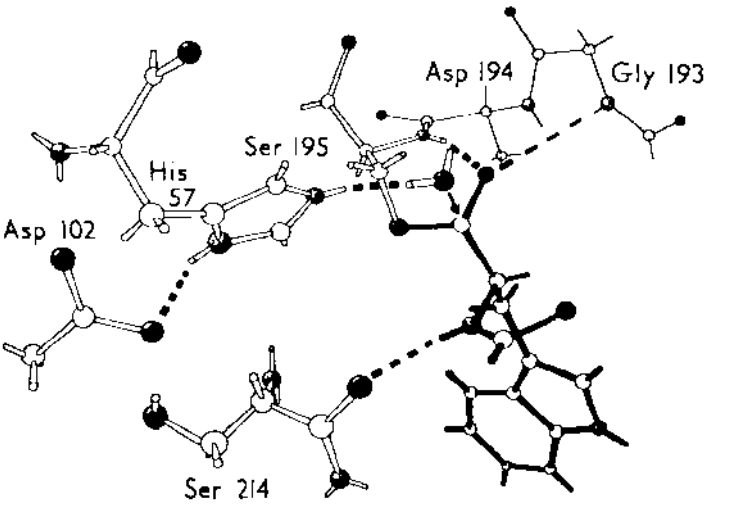


Figure 5 Presumed structure of a productive or true acyl intermediate (from Reference 19).

scissile bond and that it is hydrogen bonded to serine 214. In this instance the carbonyl oxygen of the substrate can no longer form a bridging hydrogen bond to the water molecule hydrogen bonded to histidine 57. This water molecule is then in a position for nucleophilic attack upon the carbonyl carbon of the acylating substrate.

A crystallographic study of a true acyl intermediate that has been stabilized in the crystal at low temperature has been reported (1). However, since the resolution of this investigation was only at 3.5 Å, it is not possible to obtain any additional structural information from this study or indeed to ascertain whether the complex formed was in fact an acyl intermediate.

Enzyme Product Complex

The first complex of serine proteases whose structure was determined was that of chymotrypsin with *N*-formyl tryptophan (52). This product is a virtual substrate for the reaction, since it has been shown that the oxygen of the carboxyl is catalytically turned over. These studies showed that the specific substrate side chain is bound in a pocket adjacent to the active serine. This pocket was hydrophobic in chymotrypsin but contained an aspartic acid side chain in trypsin, thereby explaining the specificity difference between trypsin and chymotrypsin. Furthermore, it was clear from these studies that the binding of the side chain plus a formyl imido group oriented the substrate scissile bond so that it could be cleaved by the serine hydroxyl and histidine 57.

Complexes between *Streptomyces griseus* Protease A and two different tetrapeptide products have been refined at 1.8 Å resolution by James and co-workers (26). Their surprising finding is that the distance between the Ser 195 Oγ and the α-carboxyl carbon of the product is 2.58 Å in one case and 2.66 Å in the other. Since there is no continuous electron density between the Ser Oγ and α-carboxyl carbon and the distance is too large, a covalent bond is not formed; however, the distance is shorter than expected for van der Waals contact. Since the product is a virtual substrate, the situation is quite analogous to that with trypsin-PTI, where a short Oγ to carbonyl carbon distance of 2.6 Å is also observed.

On the Role of ASP 102 in Catalysis

What then is the catalytic role of the buried Asp 102 of the serine proteases? Is it anything more specific than to orient His 57 and provide a hydrogen bond acceptor? Blow et al (9) suggested that the net effect might be the partial formation of a Ser 195 alkoxide ion due to the Asp-His partially removing the serine proton. This cannot be the case in the resting enzyme, since the X-ray results show the Ser-His hydrogen

bond to be either very weak or absent. Kraut et al (33, 37) focused attention on the Asp-His role in providing a pathway for the serine proton as it moves from the serine to the amide of the substrate's scissile bond. They suggested that the role of the Asp-His is not to increase the nucleophilicity of the serine but rather to effect the protonation of the scissile bond in the tetrahedral intermediate. As we see below, a similar role for the Asp-His pair had been proposed (48) from NMR studies of boronate complexes, which are analogs of the tetrahedral intermediate.

If the charge relay is intact in the pretransition state, as argued above, then it presumably would be intact, under similar conditions, in papain (2). In this case we suggest that the coordinated charge of two residues, i.e. *Asp*-His-*Ser* to *Asn*-His-*Cys*, provides the same balance of forces on the proton and that the strong proton-attracting negative charge of the buried Asp, needed to help ionize the Ser, is not needed to ionize the same acidic Cys. Our argument suggests that in the PTI-papain complex, if it could be formed, the histidine would also be positively charged, as could be determined from a C_2-H proton NMR experiment.

The other coordinated proton transfer in our present model is in the tetrahedral complex (discussed below). In that step there also is the possibility of cooperative Asn-Cys interaction in place of the Asp-Ser.

Possible Role of Enzyme Complementarity to the Tetrahedral Intermediate

Henderson (19) first pointed out that the structure of chymotrypsin is complementary to the structure of the tetrahedral intermediate. In particular, the backbone NH groups of the Gly 193 and Ser 195 provide a binding site for the O^- formed in the tetrahedral intermediates. The ability of the enzyme to bind the tetrahedral intermediate better than the Michaelis complex could reduce the activation barrier and increase the rate of catalysis. Subsequent studies on the boronyl complexes were interpreted to be consistent with this hypothesis (36).

The possibility that the enzyme distorts or strains the substrate, either geometrically or electronically (10), towards the structure of the transition state has been a matter of serious consideration (21–23, 53) but seems to be ruled out by the NMR data. However, the stabilization of the tetrahedral intermediate in the oxyanion pocket does seem to be a way in which the structure of the substrate binding site promotes catalysis (8, 10, 20, 26, 43).

Why Are the Zymogens Not Very Active?

It appears that trypsinogen and chymotrypsinogen have enzyme activities that are 10^{-4} to 10^{-7} that of the active enzymes (28, 38) because (*a*) the

"oxyanion hole" is not present, so that stabilization of the tetrahedral intermediate is not possible and (*b*) the structure of the substrate binding site is either different or partially disordered, so that the zymogens cannot bind substrates in the orientation required for catalysis. The catalytic triad appears to be essentially in the same orientation in the zymogens and the active enzymes.

There is complete agreement among the structures of chymotrypsinogen (7, 17, 56) and the two trypsinogen structural determinations (16, 30) that the oxyanion hole, in particular the backbone NH of Gly 193, is not properly oriented to bind the oxyanion of the tetrahedral intermediate. This is due to the reorientation of Asp 194, which interacts with the imidazole of His 40 in the zymogen structures but binds the newly formed α-amino group of Ile 16 in the activated enzyme. This observation is consistent with an important role for the oxyanion hole in catalysis.

In chymotrypsinogen it is clear that the substrate binding site is not the same as in the active enzyme, particularly with regard to the specific side chain binding site (7, 41, 56). Thus, specific substrates cannot bind and be oriented, in general, as with chymotrypsin. Not surprisingly, this suggests that orientation of substrates relative to catalytic residues is important for catalysis. The situation is less clear in trypsinogen, since two structures have been obtained that differ in the details of the substrate binding site. Huber and co-workers (16) do not see electron density for a portion of the substrate binding site, the "activation domain" in their $2F_o-F_c, \alpha_c$ Fourier calculated at 1.8 Å using refined coordinates. In an isomorphous replacement map at 1.9 Å resolution, Stroud and co-workers (30) see density, although weaker, for polypeptide in the activation domain; the largest differences between the trypsin and trypsinogen are in this region. Some increased flexibility and disorder in this region seems likely; however, the difference between the two results has not been completely resolved. An important difference between the two studies may be the inclusion of low resolution data in the Stroud study, which would be important for atoms with high effective thermal parameters. The differences in interpretation of the two structures notwithstanding, it appears that the lack of an appropriate binding site for substrates is an important reason for the lack of activity in trypsinogen as in chymotrypsinogen.

NMR STUDIES

Native Enzyme

The properties of the catalytic triad have been the subject of several NMR studies, and the picture of the triad that emerges from these

studies is summarized below (34). In this survey we primarily review the experiments on chymotrypsin, trypsin, and particularly α-lytic protease, a small bacterial protein. The less comprehensive NMR experiments on the other serine proteases (and their zymogens) basically support the picture presented here, but these results are not discussed in detail.

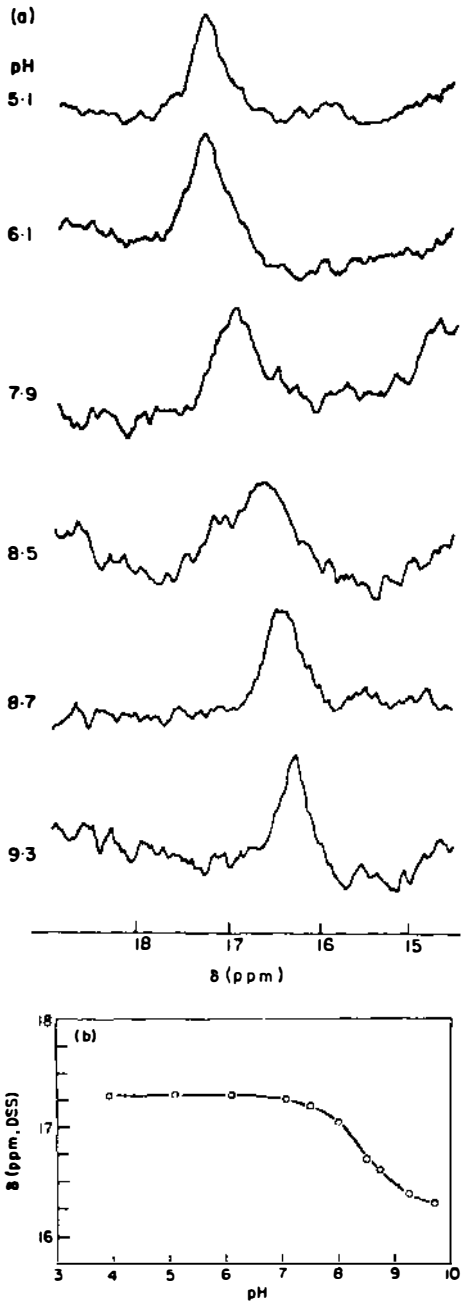
Does the Asp-His Hydrogen Bond Exist?

The most direct NMR evidence for the existence of this H-bond is the observation, originally by Robillard & Shulman, of a low field proton NMR peak that was assigned to this H-bonded proton (45–48). This broad resonance was only observed in H_2O solution, disappearing in D_2O because of exchange with the solvent. Although in the enzyme the peak was lost, presumably because of exchange broadening as the pH went through the apparent pK_a of 7.5 at 3°C, in the zymogen it could be followed continuously; both titrations are plotted in Figure 6. Aside from the slow exchange jog at low pH, the enzyme and the zymogen are identical. This assignment to the Asp-His hydrogen bond is based upon a number of experimental tests and, although originally contested (24), is now generally accepted. A resonance in this spectral region, titrating with a pK near 7, has been observed in all the serine proteases where it has been sought: chymotrypsin A, chymotrypsinogen A, trypsin, trypsinogen, subtilisin BPN, and α-lytic protease. However, it has not been noted in any protein without a charge relay system. The existence of this resonance provides clear support for the existence of the Asp-His hydrogen and also monitors its properties. A bound proton resonance is observed only when its exchange rate with H_2O is slowed sufficiently so that it can be observed. This means a residence time >3 msec., a time that is readily maintained by a hydrogen bond but that would be generally shorter without such bonding.

Two chemical modifications resulted in a loss of this -NH resonance. These were the *N*-methyl derivative of His-57 and the tosylated derivative. Crystal structures of both these derivatives had been determined, and in both cases structural changes had been reported that would weaken or eliminate the His-Asp hydrogen bond (6, 55).

NMR evidence for the existence of the His-Ser hydrogen bond is not unequivocal and is discussed below.

Figure 6 (*a*) 220 MHz ^1H NMR spectra of the low field N-H peak of bovine chymotrypsin A_δ in ^1H_2O. Enzyme concentration was 3 to 4 mM in buffered solutions of ionic strength 0.5, 3°C. (*b*) Comparison of the titration curves of the low field N-H of chymotrypsin A_δ (*squares*) shown above and of bovine chymotrypsinogen A (*crosses*). The dashed line represents a theoretical titration curve with a pK_a of 7.5. [From Robillard & Shulman (47).]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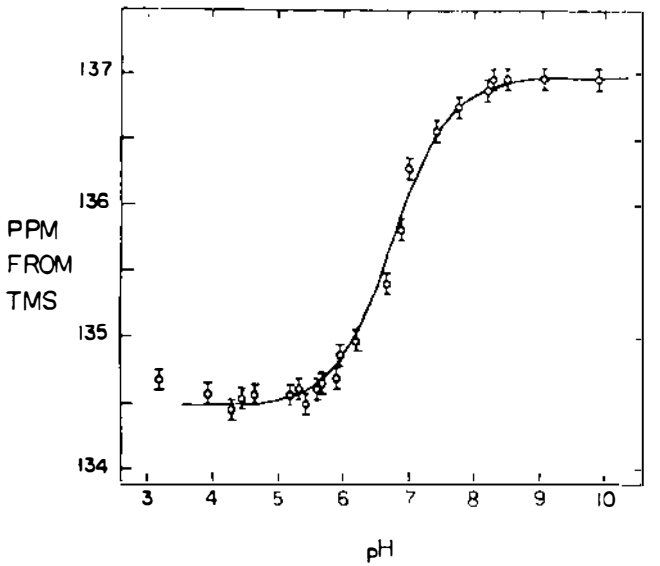

Figure 7 ^{13}C NMR titration of α-lytic protease from Hunkapiller et al (24).

Charge Distribution in the Triad

Certain particularly informative NMR experiments help to determine the charge distribution in the resting state of the enzyme. Since this kind of experiment has been particularly successful in α-lytic protease, we discuss this enzyme intensively. This small ($M_r = 19{,}869$) bacterial enzyme contains one histidine, which is in the active site triad. Hunkapiller et al (24) grew the soil bacterium Myxobacter 495 on enriched 2-^{13}C histidine and measured the ^{13}C resonance of the resting enzyme as a function of pH. They observed a resonance from the labeled histidine that titrated at a pK 6.75 at 34°C and that shifted from 137 ppm to 134.5 ppm downfield from tetramethylsilane (TMS) going to low pH. The results of this titration (shown in Figure 7) fit very well with a pK_a of 6.75 and a $\Delta\delta$ of 2.46 ppm.

Hunkapiller et al (24) also measured the coupling J_{CH} of the C_2 carbon and proton and reported no change in its value of 205 Hz until the pH was decreased to 3.5, at which point the ^{13}C peak split into three different peaks, one of which had $J_{CH} = 218$ Hz, characteristic of a protonated histidine. Unfortunately they chose to base their explanation of the charge state on the coupling constants. They proposed that the imidazole's charge remained constant and neutral, while going through the region of the pK_a of 6.75, and that this was achieved by a double proton transfer—one onto the histidine N_1 and the other from the His N_3

to the aspartate. This gave the Asp a pK_a of 6.75 and assigned the His a very low pK_a of 3.5. This novel view of the charge state, which disagreed with chemical experience and Robillard & Shulman's interpretation of their NMR results, remained an active controversy until recently. Bachovchin et al have repeated these ^{13}C-labeled histidine experiments on a high sensitivity superconducting NMR spectrometer and have found that, contrary to the early report, the J_{CH} of His C_2 does change exactly as expected for a titrating imidazole i.e. from 206 Hz at pH 8 to 217 Hz at pH 6.0 (3). This removes the early objections to a histidine titration with pK_a of 6.75, and now both the ^{13}C chemical shift and coupling constants are consistent with a normal histidine titration in the vicinity of 6.75.

In part, this clear picture of a histidine titration was achieved earlier by Bachovchin & Roberts (4) who substituted ^{15}N-enriched imidazole for the histidine and concluded from the chemical shift changes, which titrated with a pK_a of 7.0 ± 0.1 at 26°C, that the histidine was being protonated in a normal fashion.

The magnitude of the ^{15}N chemical shift change introduced by protonation was determined by comparison with several model compounds. A complication is introduced by the existence of two nitrogens, either of which might be the titrating group. By comparison with the model compounds such as 3-methylimidazole, they were able to show that in α-lytic protease above the pK_a the tautomer with hydrogen on N_1 of the imidazole ring predominates. By comparison with simple 5-substituted imidazole derivatives and with histidyl residues in other proteins they emphasized that the protonated N_1 was not the usual tautomer. Bachovchin & Roberts suggested from these results that the predominance of the protonated N_1 tautomer in α-lytic protease is caused by a hydrogen bond between the NH at the N_1 position and the "buried" carboxylate of the aspartic acid in the triad.

Two additional titrations of protons on the imidazole ring in α-lytic protease have recently been made by Markley and his associates (34, 34a) which are basically in agreement with the ^{13}C and ^{15}N results. In the first series of experiments they titrated the low field exchangeable NH proton. This had previously been observed at pH3 by Robillard & Shulman and assigned to the hydrogen-bonded proton partially by analogy with their more complete results on this low field resonance in chymotrypsin. The full titration has been obtained for α-lytic protease by Markley et al (Figure 8), who obtained a good fit to a pK_a of 6.9 at 3°C. One particular value of this observed titration is that it removes the complication introduced by Hunkapiller et al (24) who reported that they had not been able to observe the low field NH resonance in α-lytic protease, which had previously been reported (47).

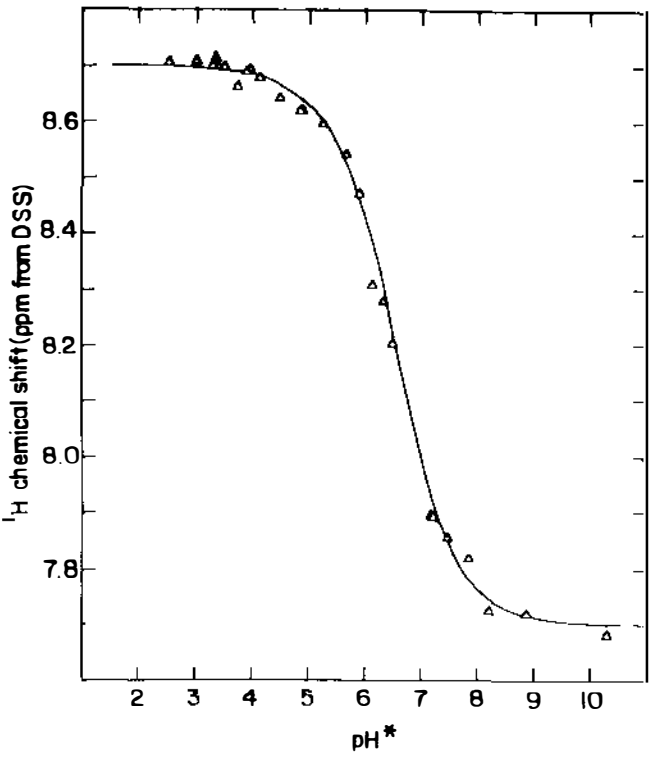

Figure 8 Titration of the C_2-H proton in α-lytic protease [from Westler & Markley (54)].

The second significant contribution by Markley et al has been the observation and titration of the C_2-H proton resonance of α-lytic protease obtained at 360 MHz using resolution enhancement techniques after exhaustive exchange of non-covalently bound protons with deuterons (see Figure 8). Excellent measurements of the C_2-H proton resonance obtained in this careful experiment are given in Figure 9, where the pK_a is 6.5 ± 0.1. [Markley has pointed out (34) that their earlier report of a lower value of this pK_a was in error because of a pH electrode miscalibration and is to be ignored.]

Charge State of Histidine

The NMR results on α-lytic protease presented in the previous section provide the basis for an unequivocal view of the degree of protonation of the histidine in the resting state of the enzyme as a function of pH. These conclusions are summarized in this section. Table 2 summarizes the measured pK_a values discussed above. It is clear that all these values

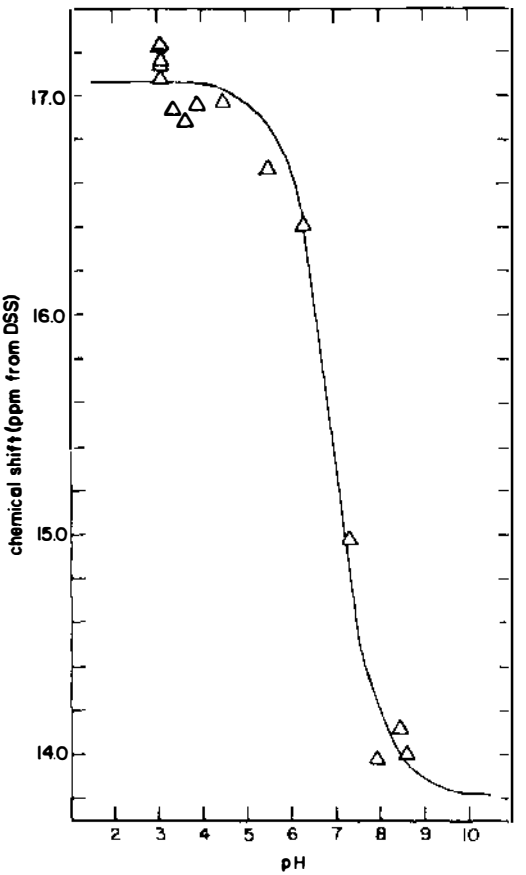

Figure 9 Titration of the NH hydrogen-bonded proton in α-lytic protease [from Westler & Markley (54)].

agree very well. The low value of 6.5 for the C_2-H proton resonance should be increased by 0.4 pH units to account for the H_2O calibration being used for this D_2O measurement. Hence, pK_a 6.9±0.1 is consistent with all four of these different measurements.

What is the charge on the imidazole ring in the enzyme above and below the pK_a? The answer is that, to within the experimental errors, the imidazole ring goes from neutral to fully protonated at its pK_a. This conclusion is based on the NMR chemical shifts whose changes upon protonation, $\Delta\delta$, can be calibrated in reference systems. These values for ^{13}C, ^{15}N, and the C_2-^1H all give results for the protein that indicate approximately a full proton has been added to the imidazole. The values

Table 2 The observed values of pK_a for α-lytic protease [a]

Resonance measured	pK_a measured	Temp. °C	Solvent
$^{15}N_1$ and $^{15}N_3$	7.0	26°	H_2O
$^{13}C_2$	6.8	34°	H_2O
C_2-^1H	6.5	30°	D_2O
N-^{1}H-Asp	6.9	3°	H_2O

[a] The C_2-^1H pK_a was measured with a glass electrode in D_2O after being calibrated in H_2O.

Table 3 Values of $\Delta\delta$ [a]

Resonance	α-Lytic protease	Model system	Reference
$^{13}C_2$	2.46	2.28	24
C_2-^1H	1.00	1.05	54
$^{15}N_1 + {}^{15}N_3$	29.2	32.2	4

[a] The reference for $^{13}C_2$ is the average of five model compounds measured by Hunkapiller et al (24); for the C_2-^1H it is the average for N_1-CH_3-histidine and N_3-CH_3-histidine (54); for the ^{15}N it is an average of the values obtained in five model compounds by Bachovchin & Roberts (4).

The C_2-^1H calibration is rather arbitrary because in other proteins and model systems different values of $\Delta\delta$ are observed, usually smaller. Generally this can be rationalized because distant contributions to the chemical shifts can be several tenths of a ppm, so that changing environments will make a larger fractional change in the protons than for the ^{13}C or ^{15}N shifts which are intrinsically much larger, while the distant contributions are the same.

are summarized in Table 3 where it can be seen that the value of $\Delta\delta$ observed in α-lytic protease ranges from slightly above the model compound values (in the $^{13}C_2$) to slightly below (in the ^{15}N results). The approximate nature of these calibrations and the small differences between the protein and the models leads us to the conclusion that the imidazole in the protein goes from unprotonated to protonated as it does in the models.

The second basis of this conclusion is the change in J_{CH} going through the pK_a, which, as mentioned above, is the same in α-lytic protease as in the model imidazole compounds.

Chymotrypsin

We now turn to the NMR results available on chymotrypsin. In addition to the NH hydrogen-bonded proton, which has pK_a 7.5 at 3°C (45), the C_2-H proton has also been followed through its pK_a by Markley and his colleagues (35, 39). In the region pH>3 there is titration of the histidine 57 C_2-^1H from 7.71 to 8.50 ppm for $\Delta\delta = 0.79$ ppm with a pK_a of 6.78. This peak was assigned to His 57 (while another histidine peak was assigned to His 40) by the fact that it no longer titrated in the

chymotrypsin—BPTI complex (50). His 57 is close to the BPTI from crystallographic studies (49) while His 40 is not, and since only one NMR peak was changed by formation of the complex the changed resonance was assigned to His 57.

Markley has pointed out that this pK_a of 6.78 measured in D_2O at 31°C is in reasonable agreement with Robillard & Shulman's value of 7.5 at 3°C, considering Hanai's reported values (18) from inhibitor-binding studies to chymotrypsin, which showed pK_a 7.2 at 3°C and 6.6 at 31°C.

The change in charge on histidine 57 upon protonation is only measured by $\Delta\delta$ of the C_2-^1H, which is 0.79 ± 0.03 ppm. As mentioned above, this is a less reliable measure of charge than the ^{13}C or ^{15}N titrations. This value of $\Delta\delta$ is consistent with many other histidine titrations and, although 20% smaller than the value observed in α-lytic protease, most probably reflects a change from neutral to positive histidines coupled to an environmental change around the histidine upon protonation.

What is the Effect of the Ser-His Hydrogen Bond?

There is no NMR experiment that directly shows whether or not a hydrogen bond exists between Ser 195 and His 57. However, there is one NMR experiment on chymotrypsin that measures the effect upon the low field N-^1H resonance of acylating the serine and thereby breaking the serine hydrogen bond if in fact it did exist (46, 47). These results on the N-^1H resonance show first that the chemical shift at low pH remained unchanged at 18.0 ppm; second, the chemical shift at high pH moved from 15.0 ppm to 14.4 ppm, and third the pK_a changed from 7.5 to 6.5. Robillard & Shulman pointed out that these results would be consistent with the existence of a hydrogen bond to serine but did not require one. The small shift upfield by 0.6 ppm upon deacylation, which could permit the serine hydrogen bond to form, is only a small fraction of the 3.0 ppm shift to lower field observed during protonation, so that the serine hydrogen bond if it existed did not appreciably protonate the histidine nor, by inference, does it allow the existence of an alkoxide ion on the serine.

It would certainly strengthen our understanding of the charge state of the resting enzyme if ^{13}C and ^{15}N measurements on the histidine could be made in stable acyl derivatives of α-lytic protease so that the charge could be more quantitatively determined. However, in view of the unequivocal crystallographic evidence against the His-Ser hydrogen bond in the resting state, we must treat the small changes observed by NMR upon acylation as reflecting a changed environment rather than breaking a hydrogen bond.

Summary of the Resting States of Chymotrypsin and α-lytic Protease From NMR Results

In summary the NMR experiments, taken together, have established with considerable certainty the following properties of the resting state of these two well-studied enzymes.

1. The hydrogen bond between His-Asp exists.
2. The histidine titrates with a normal pK_a of 6.9, and to a first approximation goes from a neutral imidazole to a fully protonated imidazolium cation.
3. From the crystallographic results it is clear that the Ser-His hydrogen bond does not exist in the resting state of the enzyme. The only available NMR evidence is ambiguous but does not disagree with the structural conclusion.

NMR Evidence on the Pretransition State Michaelis Complex

NMR experiments have been made of complexes of chymotrypsin and the trypsin inhibitors, which as described above represent a rather direct model for the Michaelis complex.

The first NMR study was of the low field N-H proton in the chymotrypsin-PTI complex (47). This resonance was observed at -14.9 ppm and, unlike the native enzyme, it did not titrate between pH4 and 8. In stable tetrahedral complexes such as benzene boronic acid and 2-phenylethyl boronic acid there were pH independent peaks at -16.3 and -17.2 ppm (47). Since the position of -14.9 ppm in the PTI complex was so far from those observed in the tetrahedral complexes, it was suggested that PTI did not form a tetrahedral complex with the imidazole ring, even though the X-ray crystal structure reports at that time favored such a tetrahedral complex.

More direct evidence for the nonexistence of a tetrahedral complex came from experiments in which the reactive site peptide bond carbons of the soybean trypsin inhibitor (5) and of the basic pancreatic trypsin inhibitor (42) have been labeled with ^{13}C and where the NMR chemical shifts have been measured in the free inhibitors and in the enzyme-inhibitor complexes. The labeled resonance was easily identified in the ^{13}C spectrum, and it shifts only 0.2 and 0.7 ppm in STI and BPTI, respectively, upon complexation. It was pointed out in both papers that this was a negligible shift compared to the shift of ~ 50 ppm expected for a tetrahedral carbon; thus it was concluded that the tetrahedral form did not exist.

With regard to the more subtle question raised by the most accurate refined crystal structures (21–23), which suggest a smaller but definite pyramidalization of the carbonyl carbon in the inhibitor, both NMR reports (5, 42) state that it is not possible to say anything about the strains observed in the scissile carbonyl carbon. We note, however, that the shifts observed upon complexation are very small.

An early experiment by Robillard and co-workers (44) measured the ^{13}C NMR of *N*-acetyl-L-tyrosine semicarbazide, which is hydrolyzed by chymotrypsin to *N*-acetyl-L-tyrosine. This was ^{13}C enriched at the carboxyl carbon. By using high concentrations of semicarbazide to maintain a high steady state level of *N*-acetyl-L-tyrosine semicarbazide, it was shown that in the Michaelis complex of this real substrate the ^{13}C resonance was shifted less than 0.1 ppm. This was interpreted as indicating that negligible substrate strain occurred in this complex at the site of enzymatic attack.

Hence, we see that very small or zero changes in the substrate chemical shifts were observed in all of the Michaelis complexes studied by NMR i.e. the two trypsin inhibitors and the real substrate, *N*-acetyl-L-tryosine semicarbazide. These results qualitatively do not support the suggestion of a strained substrate. However, there is no calibration of either aspect of this strain hypothesis to enable us to say quantitatively that substrate strain is not a contributing factor, although there is no experimental support for this idea, as discussed.

Another interesting aspect of the results on the trypsin inhibitor complexes is the possibility that the NMR shifts can give information about this state of enzyme, where the charge relay triad is complete. Two resonances have been measured in the chymotrypsin-PTI complex: the N-H hydrogen bonded proton has been observed at -14.9 ppm, essentially the position it has in the unprotonated enzyme. On the other hand the C_2-^1H proton is at -8.48 ppm, which is very close to the position it has in the low pH protonated form of the enzyme. Hence, there is a significant difference between the charges that are correlated with these shifts. In addition, we note that in porcine trypsin Markley and Porubcan have measured the C_2-H proton resonance of His-57 in the free enzyme and in the PTI complex. Its position is also very close to that observed in the protonated enzyme, and the authors conclude that His-57 is positively charged in the trypsin-PTI complex. It would be particularly important for these experiments to be extended and if possible to be done on similarly complexed α-lytic protease that has been labeled with ^{13}C or ^{15}N. However, given the definite correlation of the C_2 proton resonance with charge, and the way it shifts in both the trypsin and chymotrypsin complexes, we feel that the NMR results must be inter-

preted, following Markley et al (34, 34a) as showing the existence of a protonated imidazole in the trypsin inhibitor complexes. This implies the possible existence of a serine alkoxide ion. Crystallographic results (21–23) show the existence of a properly orientated Ser-His hydrogen bond. Since the histidine is protonated (from the NMR results), it presumably has received a proton from the His-Ser hydrogen bond. It is not known for certain whether the trypsin-PTI complex is protonated, presumably at the serine. However, the crystallographic results appear to be less consistent with a protonated than with an unprotonated serine. There is little space for an additional proton to be bound to Ser 195 Oγ, which is tightly packed against the inhibitor (21–23). Furthermore, the binding studies of Vincent & Lazdunski (53b) suggest from their pH dependence, that the inhibitor binds more strongly to the nonprotonated enzyme.

The shift of the NH proton cannot presently be used as a measure of imidazole charge, since it has no calibrations in model systems. Situated as it is, in a hydrogen bond, its position could be very sensitive to small structural charges, which would not necessarily correlate with charges on the imidazole ring.

NMR Studies of Tetrahedral Intermediates

The first stable tetrahedral intermediates we discuss are the diisopropylphosphoryl (DIP) derivatives of chymotrypsin and chymotrypsinogen. These derivatives were used by Markley & Ibanez (35) and by Porubcan et al (40) to assign the C_2-H proton resonances in these proteins. These particularly interesting experiments showed that at high pH the C_2-^{1}H resonance of His-57 had identical chemical shifts with or without DIP, whereas at low pH the shift in chymotrypsinogen was 9.03 ppm with DIP and 9.22 ppm without DIP. Taking these proton shifts as measures of charge, one could estimate that in the DIP complex the positive charge on the imidazole is ~75% as large as it was without DIP, although these small changes could have other causes.

The ^{31}P NMR studies of the DIP in the chymotrypsinogen complex showed a shift of 0.2 ppm to higher field upon protonation, indicating that approximately the same amount of positive charge missing from the imidazole in the presence of DIP was to be found on the phosphate group. The neutron diffraction of the trypsin-DIP complex at low pH showed that two protons were on the imidazole, one of which was hydrogen bonded to the phosphate (31, 32). The NMR ^{31}P shifts suggest that this proton is not as close to the nitrogen of the imidazole ring as it would be in absence of DIP.

The existence of a hydrogen bond between imidazole and the tetrahedral group, and the interaction of that tetrahedral group upon the

charge of the imidazole, has also been observed by NMR studies of a series of stable tetrahedral borate complexes with chymotrypsin (47). In the complexes with borate, benzene boronic acid, and 2-phenylethyl-boronic acid the positions of the N-^{1}H hydrogen-bonded proton, were 15.9, 16.3, and 17.2 ppm respectively. It was suggested from these results that the proton hydrogen bonded to the tetrahedral compound was shared differently between the borate and the imidazole, depending upon the electronic and structural properties of the tetrahedral group. This sharing was showing up as different positions of the N-H proton position on the other side of the imidazole. The hydrogen bond between imidazole and the tetrahedral group was described as having a more shallow potential minimum for the proton than the usual hydrogen bond, as judged by it having different effects upon the N-H resonance on the other side of the imidazole. It was also suggested that the flexible energetic response of this proton would allow it to accommodate to the energy demands of the peptide bond (or its analog) as it stretched toward breaking. Hence it would catalyze the hydrolysis of the tetrahedral intermediate. On this basis we see a role for the His-Asp section of the charge relay in providing the His-tetrahedral N-H proton with a larger electronic buffering system than that provided by the imidazole, alone. Furthermore, it is clear that in papain both Asn and Cys would be acting on this proton, presumably with similar results. Because the His-Asp N-H proton resonance responds to both charge and structure, it is not a very reliable indicator of these postulated varying imidazole charge states. Now that the C_2-H proton resonance has been observed in trypsin and chymotrypsin, it would be a very strong test of this model to measure its position in these different boronates. The prediction of this model from the NH proton shifts is that there would be increasing shifts to lower field of the C_2-H proton, going from borate to benzene boronic acid to 2-phenylethyl boronic acid.

DISCUSSION AND SUMMARY

Our survey of the crystallographic and NMR experiments suggests models of the charge relay triad for several stages of the well-established enzymatic pathway of the serine proteases. These are as follows.

First, in the resting state of the enzyme the His-Asp hydrogen bond exists but the His-Ser does not.

Second, in the resting state the imidazole ring goes from neutral to fully protonated with a pK_a of 6.9 ± 0.1 in α-lytic protease and with similar values in the other enzymes.

Third, in complexes with the trypsin inhibitor (soybean or pancreatic) NMR results show the inhibitor's scissile bond is not tetrahedral and the NMR peaks are not appreciably shifted from those in the planar, uncomplexed inhibitor. Similar conclusions, indicating no substrate strain, are obtained from ^{13}C NMR studies of the Michaelis complex of a real substrate, *N*-acetyl-L-tryosine semicarbazide.

Fourth, in the trypsin inhibitor complexes, water molecules are excluded from the active site and crystallographic results show the existence of the His-Ser hydrogen bond. In this complex the C_2-H proton NMR shifts show that the imidazole ring is protonated. The most likely interpretation of the crystallographic results is that the proton probably comes from the serine. The possibility that the system has been protonated leaving a proton on the serine seems less likely, given the close packing of inhibitor atoms around the serine Oγ and the pH dependence of binding (53b). Hence in this complex, which approximates a Michaelis complex, the charge relay system seems to be operating and the serine is, by inference, close to being an alkoxide ion.

Fifth, in the stable tetrahedral complexes, which are analogs of the tetrahedral intermediate, a hydrogen bond exists from the nitrogen of His 57 to an oxygen of the borate or phosphate compound. This oxygen is analogous to the peptide nitrogen of the scissile bond. The charge of the hydrogen in this hydrogen bond is distributed between the imidazole and the tetrahedral group, and the degree of this charge distribution depends upon the nature of the tetrahedral group. It is proposed that this proton is so flexible because of the electronic buffering provided by the His-Asp part of the charge relay and that this flexibility influences the rate of peptide bond cleavage.

The most crucial point being suggested, which is the evidence for an alkoxide form of serine (discussed in the fourth point), depends upon the NMR results of Markley and his colleagues and upon the crystallography done by Huber and his colleagues. The existence of the Ser-His bond was shown by Huber et al, while the positive charge on the imidazole ring under these conditions was stated by Markley as the explanation of his results. We propose several experiments that would test the hypothesis that these results indicate the formation of the charge relay.

Neutron diffraction studies of the trypsin-PTI complex could locate the protons in the triad and determine whether an alkoxide ion exists and if the histidine is in fact protonated.

It is possible to form the anhydrotrypsin-PTI complex and it should be possible to measure the C_2-H proton resonance to see whether the histidine, in this case, was neutral.

Third, it would be particularly desirable to measure the histidine's charge by NMR in steady state enzyme complexes with the product or with a substrate, such as *N*-acetyl-L-tryosine semicarbazide. In particular it would be desirable to measure the ^{13}C or ^{15}N positions in α-lytic protease, in one of these complexes, to give an even more reliable indication of the histidine's charge in an analog of the Michaelis complex.

If this interplay of spectroscopy and structure turns out to have added to our understanding of this mechanism, our hopes in writing this review will have been justified.

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