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^{15}N NMR Spectroscopy of Hydrogen-Bonding Interactions in the Active Site of Serine Proteases: Evidence for a Moving Histidine Mechanism†

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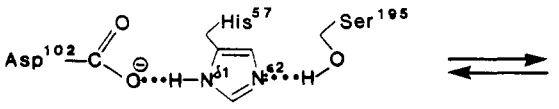
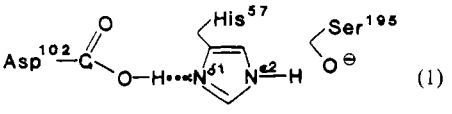
ABSTRACT: Nitrogen-15 NMR spectroscopy has been used to study the hydrogen-bonding interactions involving the histidyl residue in the catalytic triad of α-lytic protease in the resting enzyme and in the transition-state or tetrahedral intermediate analogue complexes formed with phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate. The ^{15}N shifts indicate that a strong hydrogen bond links the active site histidine and serine residues in the resting enzyme in solution. This result is at odds with interpretations of the X-ray diffraction data of α-lytic protease and of other serine proteases, which indicate that the serine and histidine residues are too far apart and not properly aligned for the formation of a hydrogen bond. In addition, the nitrogen-15 shifts demonstrate that protonation of the histidine imidazole ring at low pH in the transition-state or tetrahedral intermediate analogue complexes formed with phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate triggers the disruption of the aspartate–histidine hydrogen bond. These results suggest a catalytic mechanism involving directed movement of the imidazole ring of the active site histidyl residue.

The active sites of serine proteases invariably contain a particular arrangement of the side-chain functional groups of aspartic acid, histidine, and serine known as the "catalytic triad" or "charge-relay" system (**1a**). How this triad functions



1a



(1)

1b

in catalysis is an old problem, which despite extensive effort has not been solved.

Central to the problem is the question of whether or not a H-bond[1] links $N^{\epsilon 2}$ of His-57 and O^{γ} of Ser-195 in the resting enzyme at catalytically active pH (>7.0). X-ray diffraction studies of chymotrypsin (Blow et al., 1969) and of other serine proteases (Kraut, 1977) were first interpreted as indicating that this H-bond is formed, along with a second H-bond between His-57 and Asp-102 as shown in **1a**. This finding of a H-bonded network linking Asp-102, His-57, and Ser-195 prompted Blow to postulate his charge-relay theory in which he argued that the unusual reactivity of the active site serine—the hallmark of serine proteases—could be understood in terms of the generation of a serine alkoxide ion (**1b**). Negative charge, according to this scheme, is in effect transferred from the carboxylate anion of Asp-102 to the serine via the H-bonding network (eq 1).

Blow's charge-relay theory, as originally formulated and understood by others in the field, had a number of deficiencies and did not gain much support. However, a modified charge-relay scheme proposed by Hunkapiller and co-workers (Hunkapiller et al., 1973) did gain wider acceptance. In this scheme, attack of the serine hydroxyl group on the carboxyl carbon of the substrate is accompanied by the concerted transfer of two protons: the serine hydroxyl proton to $N^{\epsilon 2}$ of His-57 and the $N^{\delta 1}$ proton of His-57 to the carboxylate anion of Asp-102, as shown in eq 2. The experimental basis for this proposal came from a measurement of the spin coupling between the histidyl's $C^{\epsilon 1}$ carbon and its attached proton, which indicated that the imidazole ring had an abnormally low pK_a—lower in fact than that of the carboxylate of Asp-102,

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[1] Abbreviations: H-bond, hydrogen bond; PMSF, phenylmethanesulfonyl fluoride; DIFP, diisopropyl fluorophosphate; TI, tetrahedral intermediate; SPGA, *Streptomyces griseus* protease A; Tris, tris(hydroxymethyl)aminomethane.

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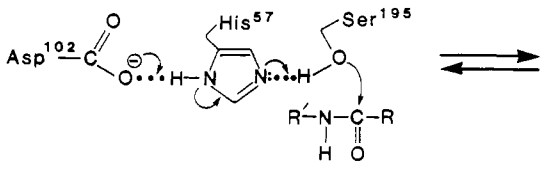
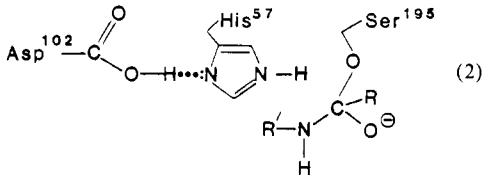


2a



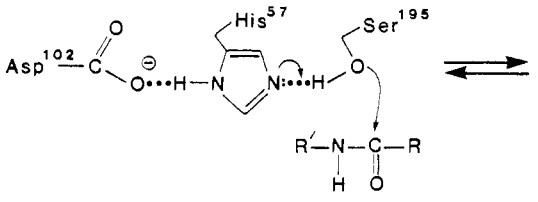
(2)

2b

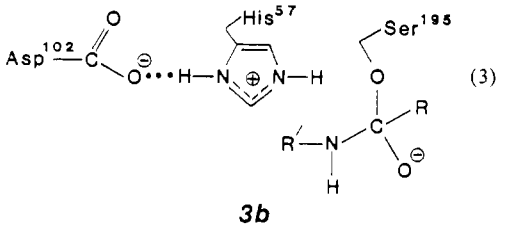
which was assigned a pK_a of 7.0. Although controversial, acceptance of this scheme was sufficient that it became incorporated into most biochemistry textbooks.

^{15}N NMR studies of His-57 in α-lytic protease first convincingly demonstrated that the modified charge-relay mechanism was untenable (Bachovchin & Roberts, 1978). ^1H NMR (Markley & Ibañez, 1978; Westler, 1980; Markley et al., 1980) and neutron-diffraction studies (Kossiakoff & Spencer, 1981) quickly supported this view. Furthermore, the coupling constant measurements of Hunkapiller and coworkers (Hunkapiller et al., 1973), which prompted them to propose their modified charge-relay theory, have been reexamined (Bachovchin et al., 1981) and have been shown to be in error.

How then does the Asp-His-Ser triad function in catalysis? The essential nature of a histidyl residue to catalysis was well established before Blow's discovery of the "buried" carboxylate of Asp-102. At that time, the histidyl residue was believed to function simply as a general-base catalyst, accepting the serine hydroxyl group's proton as it attacked the substrate. On the basis of our ^{15}N NMR results, we suggested (Bachovchin & Roberts, 1978) that the buried carboxylate of Asp-102 could enhance the histidyl residue's ability to perform this role by (i) ensuring the proper tautomeric form of His-57, (ii) orienting the imidazole ring optimally for accepting the serine proton, and (iii) providing electrostatic stabilization of the developing imidazolium cation (eq 3). His-57, of course,



3a



(3)

3b

would be more effective in providing such general base catalyzed assistance if it were strongly H-bonded to Ser-195 as shown in **3a**, a structure proposed in early X-ray diffraction studies. However, Kraut and co-workers (Matthews et al., 1977) have reexamined the X-ray diffraction data of a number of serine proteases and have concluded that $N^{\epsilon 2}$ of His-57 and O^{γ} of Ser-195 are too far apart (3.2 and 3.8 Å) and, moreover, not properly aligned for the formation of a H-bond. The criterion for the existence of this H-bond is that the distance between $N^{\epsilon 2}$ of His-57 and O^{γ} of Ser-195 be less than 3.0 Å. In the above analyses, these distances were found to be as follows: subtilisin, 3.7 Å; trypsin, 3.2 Å; α-chymotrypsin, 3.2 Å; elastase, 3.2 Å; chymotrypsin, 3.5 Å. Further support for this view, that there is little if any interaction between His-57 and Ser-195, comes from subsequent X-ray diffraction studies of α-lytic protease (Brayer et al., 1979a; Fujinaga et al., 1985) and of *Streptomyces griseus* protease A (SPGA) (James et al., 1980). These two enzymes are microbial serine proteases that are homologous with the mammalian pancreatic enzymes. In α-lytic protease, the distance between $N^{\epsilon 2}$ of His-57 and O^{γ} of Ser-195 was determined to be 3.3 Å and thus too long for the formation of a H-bond. In SPGA, however, the distance was only 2.7 Å. Nevertheless, the existence of a H-bond was ruled out on geometric grounds.

On the basis of these results, Kraut and co-workers (Kraut, 1977; Matthews et al., 1977) proposed that activation of the serine hydroxyl group by the Asp–His couple, whether by some form of a charge-relay mechanism or by a simple general-base mechanism, should no longer be considered to be an important factor in catalysis.

This issue, however, is not settled. Bode and co-workers (Bode et al., 1983) in more recent X-ray diffraction studies of kallikrein, a trypsin-like serine protease from porcine pancreas, have determined the distance between $N^{\epsilon 2}$ of His-57 and O^{γ} of Ser-195 to be 2.8 Å and thus favorable for the formation of a strong H-bond. In addition, they report that the corresponding distance in trypsin is 2.9 Å—not 3.2 Å as reported by Kraut and co-workers—and thus also favorable for the formation of a H-bond. In the most recent studies, a 1.68-Å refinement of α-chymotrypsin, Tsukada and Blow (1985) report that the $N^{\epsilon 2}$–O^{γ} distance is only 2.8 Å—not the 3.2 Å determined by Kraut and co-workers and thus favorable for the formation of a H-bond. Thus, X-ray diffraction studies are controversial on the question of whether a H-bond is formed between His-57 and Ser-195 in the resting enzyme at catalytically active pH.

Although serine proteases have been intensively studied by ^1H NMR spectroscopy, only one experiment bears on the question of H-bonding between His-57 and Ser-195. In this experiment, Robillard and Shulman (1974) found that the low-field proton signal in ^1H NMR spectra of chymotrypsin assigned to the proton in the H-bond between Asp-102 and His-57 exhibited a 0.6 ppm chemical shift change on acylating Ser-195. They pointed out that although this result would be consistent with the existence of a H-bond between His-57 and Ser-195, it did not require one.

The objective of the work reported here was to use ^{15}N NMR spectroscopy to obtain a direct and unambiguous answer to this important question. Imidazole model system studies (Schuster & Roberts, 1979; Roberts et al., 1982; Blomberg et al., 1977) have shown that nitrogen chemical shifts of imidazole ring nitrogens are quite sensitive to H-bonding interactions. In principle, ^{15}N NMR spectroscopy should be capable of supplying this type of information about histidyl residues in proteins. The results presented here confirm that this is indeed the case.

Experimental Procedures

Histidine specifically labeled with ^{15}N at $N^{\delta 1}$ (99%) was obtained from ICON (Summit, NJ). DL-Histidine ^{15}N labeled at both ring nitrogens (95%, $N^{\epsilon 2}$ and $N^{\delta 1}$) was obtained from ICON or synthesized by the method of Totter and Darby

(Totter & Darby, 1944; Darby et al., 1942). The NIH Stable Isotope Resource of the Los Alamos National Laboratory provided $^{15}NH_3$ (99%) for this synthesis. The purity and ^{15}N content of the labeled histidines were confirmed by ^{15}N and ^{1}H NMR spectroscopy. Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide was synthesized as described (Hunkapiller et al., 1976).

Incorporation of ^{15}N-labeled histidine into α-lytic protease (EC 3.4.21.12) was accomplished by culturing a histidine-requiring mutant of *Lysobacter enzymogenes* (ATCC 29487) as described previously (Bachovchin & Roberts, 1978). Enzyme activity was measured spectrophotometrically at 410 nm ($\Delta\epsilon_{410} = 8.86 \times 10^3$ M^{-1} s^{-1}) with Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide (4×10^{-4} M in 0.05 M Tris buffer, pH 8.75 at 25 °C). On the basis of $A^{1\%}_{280} = 8.9$ (Whitaker, 1970), purified preparations of α-lytic protease used in these NMR studies exhibited k_{cat}/K_m values of 2.0×10^3 M^{-1} s^{-1}.

NMR samples were prepared by dissolving lyophilized powders of α-lytic protease in 0.1 M KCl. About 10% ^2H_2O was added to provide an internal lock signal. A small amount of Tris buffer was added (0.05 M) to help stabilize the pH at high pH values.

^{15}N NMR spectra were recorded at 40.55 MHz on a Bruker AM-400 wide-bore NMR spectrometer equipped with an Aspect 2000A computer and a 10-mm single-frequency ^{15}N probe. Spectra were acquired with a 90° pulse (24 μs), a spectral width of 10000 Hz, 8K real data points, and a recycle time of 0.8 s. Chemical shifts are referenced relative to external 1 M HNO_3 in ^2H_2O with positive shifts being upfield.

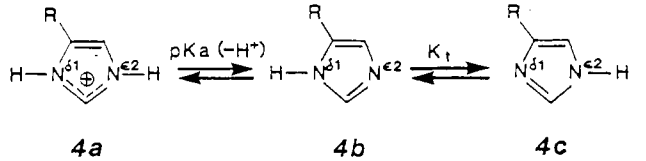
^{1}H NMR spectra were recorded at 400 MHz on the above-described Bruker AM-400 spectrometer and with a 5-mm single-frequency ^{1}H probe. Low-field resonances were resolved at low temperature (278 K) in ^1H_2O solution with the "2-1-4" pulse sequence (Redfield et al., 1975). NMR samples were maintained at 278 ± 0.5 K with the Bruker variable-temperature accessory. Spectra were acquired with a spectral width of 10000 Hz, 8K real data points, and a recycle time of 1.0 s. Chemical shifts are referenced relative to dimethylsilapentanesulfonate (DSS).

PMSF-inactivated α-lytic protease was prepared by adding a 3-fold molar excess of PMSF directly to the 2 mM α-lytic protease NMR sample at pH 9.0. Ninety percent inactivation required about 30 min at room temperature. DIFP-inactivated enzyme was prepared by adding a stoichiometric amount of predissolved DIFP directly to the 2 mM α-lytic protease NMR sample at pH 9.0. Inactivation was instantaneous.

The pH of the NMR samples was varied by the addition of 0.25 M NaOH or HCl. The pH of the solution and the activity of the enzyme were checked before and after recording each spectrum. Before and after pH measurements agreed to within 0.05 pH unit while those for enzyme activity agreed to within 5%.

Results

Model System Studies. In aqueous solutions over the pH range of biochemical interest, the imidazole ring of histidine can readily assume three basic structural forms as shown in eq 4. Structure **4a** is the protonated imidazolium ion while



(4)

4b and **4c** are tautomeric forms of the neutral imidazole ring.

Table I: Comparison of the ^{15}N Chemical Shifts of Model Systems with Those of His-57 in α-Lytic Protease[a]

compound		imidazolium cation[b]	$\Delta\delta\lvert N^{\delta1} - N^{\epsilon2}\rvert$ cation	imidazole[c]	$\Delta\delta\lvert N^{\delta1} - N^{\epsilon2}\rvert$ neutral species
imidazole		202		171	
$N^{\delta1}$-methylimidazole[d]	$N^{\delta1}$	204.1	0.5	211.5	83
	$N^{\epsilon2}$	203.6		128.5	
histidine, H_2O, 25 °C[e]	$N^{\delta1}$	200.5	2.4	143.2	55.1
	$N^{\epsilon2}$	202.9		198.3	
	$N^{\delta1}$			156.7	27.20
	$N^{\epsilon2}$			183.9	
histidine, $N^{\delta1}$–H tautomer,[f] 80% EtOH, −60 °C	$N^{\delta1}$	198.8	2.60	208.3	76.7
	$N^{\epsilon2}$	201.4		131.6	
histidine, $N^{\epsilon2}$–H tautomer,[f] 80% EtOH, −60 °C	$N^{\delta1}$	198.8	2.60	128.5	82.7
	$N^{\epsilon2}$	201.4		211.2	
$N^{\delta1}$-methylhistidine[f]	$N^{\delta1}$	203.6	0.4	210.8	76.4
	$N^{\epsilon2}$	204.0		134.4	
$N^{\epsilon2}$-methylhistidine[f]	$N^{\delta1}$	198	6.4	128.6	84
	$N^{\epsilon2}$	204.4		212.2	
α-lytic protease	$N^{\delta1}$	191.6	12.6	199.4	61.4
	$N^{\epsilon2}$	204.2		138.0	
α-lytic protease + PMSF	$N^{\delta1}$	198.0	3.50	199.7	72.1
	$N^{\epsilon2}$	201.5		127.6	
α-lytic protease + DIFP	$N^{\delta1}$	198.5	3.60	205.9	72.9
	$N^{\epsilon2}$	202.1		133.0	

[a] Chemical shifts are in ppm from external 1 M HNO_3 in D_2O. [b] Shifts under conditions of full protonation. [c] Shifts under conditions of no protonation. [d] The methylated nitrogen is arbitrarily labeled $^{15}N^{\delta1}$. [e] Shift data from Blomberg et al. (1977) and recalculated to 1 M HNO_3 standard. The first set of ^{15}N shifts for neutral imidazole is for histidine amphion; the second is for histidine anion. [f] ^{15}N shifts from W. W. Bachovchin, W. Y. L. Wong, and S. Farr-Jones (unpublished results).

According to Witanowski et al. (1972), nitrogen atoms within five-membered imidazole rings can be divided into two main types, type α in which the nitrogen atom is covalently bonded to three other atoms and type β in which the nitrogen is bonded to only two other atoms. These two types of nitrogens have very different and quite characteristic ^{15}N chemical shifts. However, type α nitrogens within protonated imidazolium rings, such as those of **4a**, generally resonate 8–10 ppm to lower field than the corresponding type α nitrogen within a neutral imidazole ring. Thus, from the standpoint of ^{15}N chemical shift ranges, nitrogen atoms within imidazole rings can be divided into three main types. Therefore, extending the nomenclature introduced by Witanowski, type α nitrogens within protonated imidazolium rings here will be referred to as type α+.

In analyzing and interpreting the ^{15}N chemical shift data from His-57 of α-lytic protease, it would be very useful to know the ^{15}N chemical shifts of the main structural forms of histidine shown in eq 4. Direct ^{15}N NMR studies of histidine in aqueous solutions at room temperature, however, do not provide the ^{15}N chemical shifts of **4b** and **4c** because these two structure rapidly interconvert under these conditions and only an averaged ^{15}N NMR spectrum is obtained (Blomberg et al., 1977).

However, *N*-methylimidazole and $N^{\delta1}$- and $N^{\epsilon2}$-methylhistidine (Table I) can be used to estimate the ^{15}N shifts of

the pure type α and type β nitrogens of **4b** and **4c** because these N-methylated derivatives do not undergo tautomerism of the type exhibited by histidine and because the replacement of a proton by a methyl appears to effect only a relative change in the ^{15}N chemical shift (Bachovchin & Roberts, 1978; Alei et al., 1980; W. W. Bachovchin, W. Y. L. Wong, and S. Farr-Jones, unpublished results). In addition, we have recently observed the pure tautomeric forms of histidine directly in ^{15}N NMR spectra of histidine in 80% EtOH–H_2O solution at low temperature (−55 °C). The agreement between the ^{15}N shifts of the pure tautomers of histidine and those of *N*-methylimidazole and $N^{\delta 1}$- and $N^{\epsilon 2}$-methylhistidine is particularly gratifying (Table I). ^{15}N NMR studies of other imidazole derivatives in aqueous solution also have yielded ^{15}N chemical shifts for pure type α, type β, and type α+ nitrogens (Roberts et al., 1982; Witanowski et al., 1972; Alei et al., 1980; Blomberg et al., 1977), which agree reasonably well with the values listed in Table I. Typical ^{15}N chemical shifts for the three main types of imidazole ring nitrogens atoms are given below for samples in aqueous solution:

nitrogen type	^{15}N chemical shift (ppm)
>N—H (type α)	210
>N (type β)	128
>N—H (type α+)	201

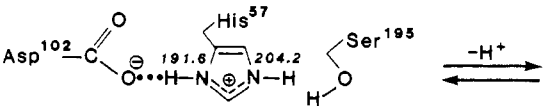
Hydrogen Bonding and ^{15}N Chemical Shifts. The imidazole ring of histidine can engage in two types of H-bonds. The type α or type α+ nitrogens can participate in hydrogen bonding as the hydrogen donor, while the type β nitrogen can participate as the hydrogen acceptor. Model system studies have demonstrated that H-bonding produces changes in the ^{15}N chemical shifts that are similar in direction to those produced by protonation/deprotonation but are much smaller in magnitude (Schuster & Roberts, 1979). Thus, a type α or type α+ nitrogen will experience a *downfield* chemical shift change of up to 10 ppm on donating its proton in a H-bond while a type β nitrogen will experience an *upfield* chemical shift change of up to 10 ppm on accepting a proton in a H-bond.

Besides having these direct effects on the ^{15}N chemical shifts of the imidazole ring nitrogens, H-bonding can also affect the observed ^{15}N shift behavior by shifting the tautomeric equilibrium. An example of this occurs with histidine (Blomberg et al., 1977). At neutral pH (8.0), the protonated α-amino group of histidine forms a H-bond to $N^{\delta 1}$ of the $N^{\epsilon 2}$–H tautomer, **4c**, effectively stabilizing this tautomer relative to the $N^{\delta 1}$–H tautomer, **4b**. Deprotonation of the α-amino group at high pH eliminates this interaction, and this results in a shift of the tautomeric equilibrium in the direction of the $N^{\delta 1}$–H tautomer, **4b**, and thus in a *downfield* chemical shift change in $N^{\epsilon 2}$ and a corresponding *upfield* chemical shift change in $N^{\delta 1}$.

Hydrogen Bonding Involving His-57. An ^{15}N NMR study of His-57 of α-lytic protease in the resting state has been reported (Bachovchin & Roberts, 1978). The ^{15}N chemical shift behavior clearly reveals the presence of the Asp–His H-bond at both ends of the titration curve of His-57.

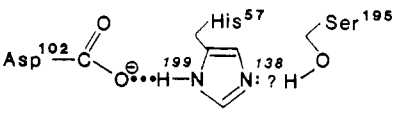
At low pH, where the imidazole ring of His-57 is protonated, $N^{\delta 1}$ and $N^{\epsilon 2}$ have ^{15}N shifts of 191.6 and 204.2 ppm, respectively (**5a**). The abnormally low-field position of $N^{\delta 1}$, 8–10 ppm from where it should be for a type α+ nitrogen, reveals the presence of a H-bond, in this case the Asp–His H-bond.

At high pH, where the imidazole ring is neutral, $N^{\delta 1}$ and $N^{\epsilon 2}$ are found at 199.4 and 138 ppm, respectively (**5b**). These ^{15}N shifts demonstrate that the $N^{\delta 1}$–H tautomer predominates. Because this is not the preferred tautomer for histidine, nor



$\Delta\delta = 12$ ppm

5a



(5)

$\Delta\delta = 61$ ppm

5b

for nearly all other 4-substituted imidazole derivatives, its predominance in α-lytic protease signals the presence of the H-bond between $N^{\delta 1}$–H of His-57 and the carboxylate of Asp-102.

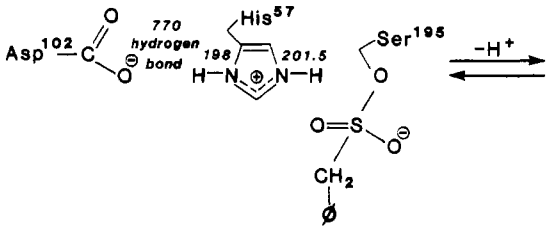
Thus, at low pH, where His-57 is protonated, the presence of the Asp–His H-bond is revealed by its effect on the ^{15}N shift of $N^{\delta 1}$ (**5a**), whereas at high pH, where His-57 is neutral, the presence of the Asp–His H-bond is revealed by its effect on the tautomeric equilibrium constant of His-57 (**5b**).

What, however, do the ^{15}N shifts reveal about the state of H-bonding between Ser-195 and His-57? From its effect on the tautomeric structure of His-57, we know that a H-bond exists between Asp-102 and $N^{\delta 1}$–H of the neutral form of His-57. However, we do not know how much of an effect this H-bond has on the ^{15}N shift of $N^{\delta 1}$–H. If we assume it has the same 8–10 ppm effect that it has on $N^{\delta 1}$ of the protonated form of His-57, then the ^{15}N shift of $N^{\delta 1}$ represents essentially that of a pure type α nitrogen, which is shifted *downfield* to its observed position at 199 ppm by the H-bond. If $N^{\delta 1}$ is essentially a pure type α nitrogen, then $N^{\epsilon 2}$ must be essentially a pure type β nitrogen because a neutral imidazole must have, on average, one type α and one type β nitrogen. The ^{15}N shift of $N^{\epsilon 2}$, however, of 138 ppm is 8–10 ppm *upfield* from where it should be for a pure type β nitrogen, indicating that it also is engaged in a H-bond, but as the hydrogen acceptor (**5b**).

An essentially equivalent way of analyzing the ^{15}N shifts is by considering the difference between the ^{15}N shifts of $N^{\delta 1}$ and $N^{\epsilon 2}$. For the neutral form of His-57, the ^{15}N shifts for $N^{\delta 1}$ and $N^{\epsilon 2}$ differ by only 60 ppm, which is ~20 ppm less than the characteristic ~80 ppm difference between pure type α and type β nitrogens. There are two possible explanations for the smaller shift difference: (1) *Tautomerism.* Exchange averaging of the ^{15}N shifts of the predominate $N^{\delta 1}$–H, **4b**, tautomer with those of a smaller amount of the $N^{\epsilon 2}$–H tautomer, **4c**, would produce equal and opposite effects on the observed ^{15}N shifts of $N^{\delta 1}$ and $N^{\epsilon 2}$, effectively moving their resonance positions closer together. (2) *Two hydrogen bonds.* As discussed earlier, the participation of a type α nitrogen in a H-bond as the donor will produce a *downfield* shift change in that nitrogen whereas the participation of a type β nitrogen in a H-bond as the acceptor will produce an *upfield* shift change in that nitrogen. Thus, both types of H-bonds tend to move the observed ^{15}N shift closer together. Of course, the 20 ppm smaller shift difference between $N^{\delta 1}$ and $N^{\epsilon 2}$ discussed above may reflect contributions from both tautomerism and hydrogen bonding. However, if 8–10 ppm of this 20 ppm is assigned to the Asp–His H-bond, the remaining 10 ppm then can only be accounted for by the presence of a second hydrogen bond to $N^{\epsilon 2}$.

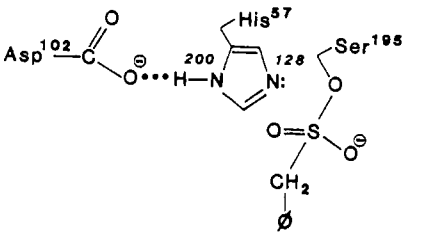
This analysis supporting the existence of a His–Ser H-bond depends critically upon the assumption of an 8–10 ppm ^{15}N shift change in N^{δ_1}–H because of H-bonding to Asp-102. If the effect were much less, then tautomerism rather than two H-bonds might explain the ^{15}N shifts observed for the neutral form of His-57.

However, this can be experimentally tested. The possibility of His–Ser H-bonding can be eliminated by specific covalent modification of Ser-195. If a His–Ser H-bond does exist in the resting enzyme, modification of Ser-195 should result in a 8–10 ppm *downfield* change in the ^{15}N shift of N^{ϵ_2} with little or no change of the ^{15}N shift of N^{δ_1} (assuming that the H-bond is not important in maintaining the tautomeric structure of His-57). Figure 1 shows the results of this experiment. Sulfonylation of Ser-195 (eq 6) with phenylmethanesulfonyl



$\Delta\delta$ = 3 ppm

6a



(6)

$\Delta\delta$ = 72 ppm

6b

fluoride (PMSF) at pH 8.5 does indeed produce a 10 ppm downfield shift change in N^{ϵ_2} (compare **5b** and **6b**) from 138 ppm in the resting enzyme (Figure 1, bottom) to 128 ppm in the modified enzyme (Figure 1, middle). The ^{15}N shift of N^{δ_1} changes only slightly, moving to 199.7 ppm in the PMSF-inhibited enzyme from 199.4 in the resting enzyme. (Note that in the PMSF-inhibited enzyme the ^{15}N shifts of N^{δ_1} and N^{ϵ_2} are now ~72 ppm apart compared to ~61 ppm in the resting enzyme; this also supports the view that one H-bond has been eliminated but that one still remains.) Essentially the same result is obtained when diisopropyl fluorophosphate (DIFP) is used to phosphorylate Ser-195. (The ^{15}N signals are much broader in the DIFP-modified enzyme for reasons that are not yet entirely clear.)

These results strongly support the existence of a H-bond between N^{ϵ_2} of His-57 and Ser-195. They also demonstrate (i) that the imidazole ring of His-57 exists nearly exclusively as the N^{δ_1}–H tautomer in the resting enzyme and in the PMSF- and DIFP-inactivated enzymes and (ii) therefore that the Asp-102–His-57 H-bond alone is sufficient to maintain His-57 in this tautomeric form.

Lowering the pH of the PMSF- and DIFP-inactivated enzymes sufficiently to protonate His-57 (pH 4.0) produces a suprising result (Figure 2). In both inactivated enzymes, N^{δ_1} of His-57 resonates at 199 ppm, approximately 8 ppm *upfield* from its abnormally low-field position of 191.6 ppm in the

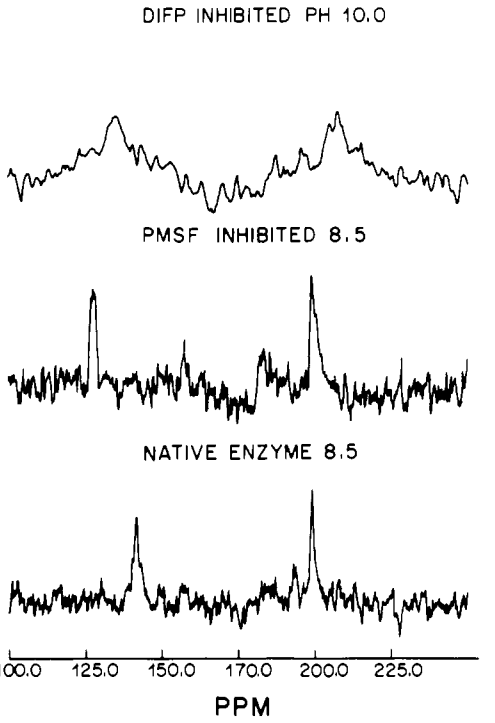


FIGURE 1: ^{15}N NMR spectra (40.55 MHz) of ^{15}N-labeled His-57 α-lytic protease (95% ^{15}N, both ring nitrogens) under conditions where His-57 is fully neutral. Enzyme concentrations were about 2 mM for each spectrum. The spectrum of the native enzyme (bottom trace) and of the PMSF-inactivated enzyme (middle trace) each represent about 10 000 scans collected with a acquisition time of 0.2 s and a recycle time of 0.8 s. The spectrum of the DIFP-inactivated enzyme required about 130 000 scans because of the extreme width of the NMR lines.

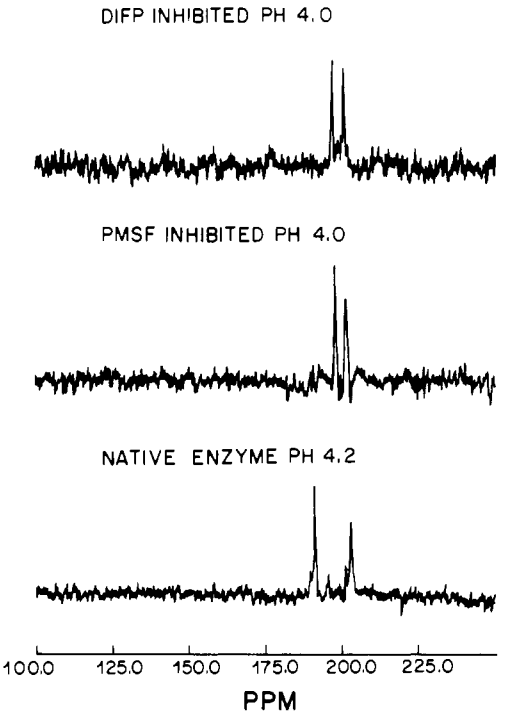


FIGURE 2: ^{15}N NMR spectra (40.55 MHz) of ^{15}N-labeled His-57 α-lytic protease (95% ^{15}N, both ring nitrogens) under conditions where His-57 is fully protonated. Enzyme concentrations were about 2 mM, and each spectrum represents about 10 000 scans collected with an acquisition time of 0.2 s and a recycle time of 0.8 s.

resting enzyme, which is diagnostic of the H-bond to Asp-102. The $^{15}N^{\delta_1}$ shift of 199 ppm, typical for a type α+ nitrogen not engaged in a H-bond, demonstrates that protonation of His-57

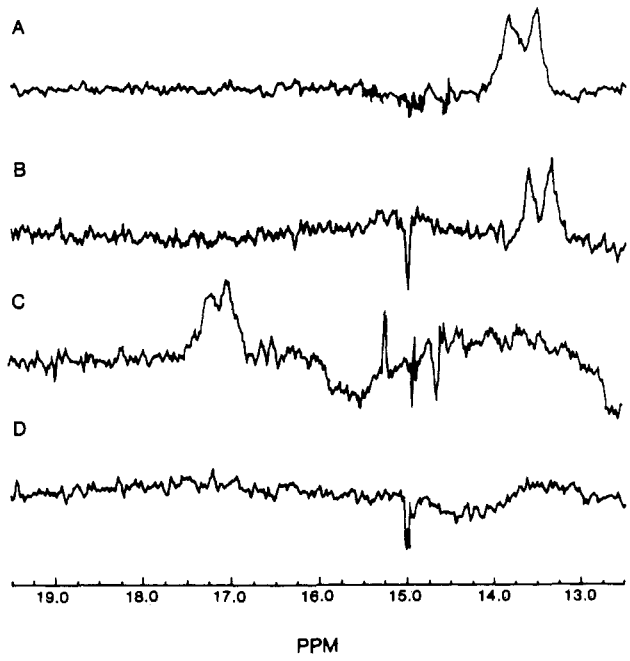


FIGURE 3: Low-field region of the 400-MHz ^1H NMR spectrum of $^{15}N^{\delta 1}$-labeled His-57 α-lytic protease: (A) native enzyme, pH 9.0; (B) PMSF-inactivated enzyme, pH 9.0; (C) native enzyme, pH 4.0; (D) PMSF-inactivated enzyme, pH 4.0. Spectra A–C each represent about 1000 scans while spectrum D represents about 2000 scans. Enzyme concentations were about 1 mM for all spectra.

in the PMSF- and DIFP-inactivated complexes triggers the disruption of the Asp–His H-bond (compare **5a** and **6a**). (Note in Figure 1 how the line widths of the DIFP-inactivated enzyme at pH 4.0 are approximately the same as those of the PMSF-inactivated enzyme. Thus, whatever it is that severely broadens the ^{15}N signls of the DIFP-inactivated enzyme at high pH is eliminated by lowering the pH to 4.0.)

Thus, the ^{15}N shifts demonstrate that in the PMSF- and DIFP-inactivated enzymes a strong H-bond links Asp-102 and His-57 when His-57 is neutral but that this H-bond is broken when His-57 becomes protonated. The effect is fully reversible. Therefore, the disruption of the H-bond cannot be due to denaturation of the PMSF- and DIFP-modified enzymes at low pH.

Low-Field Proton NMR. The conclusion that the Asp–His H-bond breaks in the PMSF- and DIFP-inactivated enzymes when His-57 becomes protonated can be tested by ^1H NMR spectroscopy. Proton NMR spectra of α-lytic protease and other serine proteases (Robillard & Shulman, 1974; Bachovchin, 1985) show a single low-field resonance (14–18 ppm) in ^1H_2O solution at low temperatures (5 °C). This resonance has been assigned to the proton in the H-bond between Asp-102 and His-57. The abnormally low-field chemical shift of this N–H proton and its slow exchange rate with ^1H_2O are due to its location in the Asp–His H-bond. Thus, disruption of this H-bond should result in the disappearance of the low-field resonance from the ^1H NMR spectra.

Figure 3 shows the results of this experiment. Spectra A and C essentially reproduce the work of Robillard and Shulman (1974) by showing the presence of a low-field proton resonance in spectra of native α-lytic protease, which titrates from 13.9 ppm at pH 9.0 to 17 ppm at pH 4.0. Here the resonance appears as a doublet because the enzyme used for these experiments was $^{15}N^{\delta 1}$ of His-57. (The observation of spin coupling to ^{15}N confirms the assignment of this proton resonance.) Spectrum B, by showing the presence of a low-field resonance centered at 13.5 ppm, confirms the presence of the Asp–His H-bond in the PMSF-inactivated enzyme at pH 9.0, where His-57 is neutral, while spectrum D shows that a low-field signal is *not* detected in spectra of the PMSF-inactivated enzyme at pH 4.0, where His-57 is protonated. These results confirm the conclusions reached in the ^{15}N studies that the Asp–His H-bond is maintained in the PMSF- and DIFP-inactivated enzymes at high pH, where His-57 is neutral but that protonation of His-57 in these tetrahedral intermediate analogue complexes triggers the disruption of the Asp–His H-bond.

Note that in the PMSF-inactivated enzyme at pH 9.0 (Figure 3B) the low-field resonance has moved upfield 0.4 ppm from its position in the resting enzyme to 13.5 ppm. This agrees closely with the finding of Robillard and Shulman (1974) of a 0.6 ppm upfield movement of the low-field resonance of chymotrypsin on acylating Ser-195 with *p*-nitrophenyl N^2-(*N*-acetylalanyl)-N^1-benzylcarbazoate, a compound that reacts with chymotrypsin to form a stable acyl enzyme. Although Robillard and Shulman (1974) originally interpreted their result as support for the existence of a His–Ser H-bond, the X-ray diffraction evidence against a His–Ser H-bond caused Steitz and Shulman (1982) to reinterpret this result as "reflecting an environmental change rather than breaking a H-bond". The ^{15}N and low-field proton results on PMSF-inactivated α-lytic protease described here indicate that the original interpretation was correct.

Discussion

His–Ser Hydrogen Bonding and Charge Distribution. The results reported here demonstrate that for α-lytic protease in the resting state in solutions of high pH the imidazole ring of His-57 exists exclusively as the $N^{\delta 1}$–H tautomer and, moreover, that both imidazole ring nitrogens participate in strong H-bonds, $N^{\delta 1}$–H as a donor and $N^{\epsilon 2}$ as an acceptor.

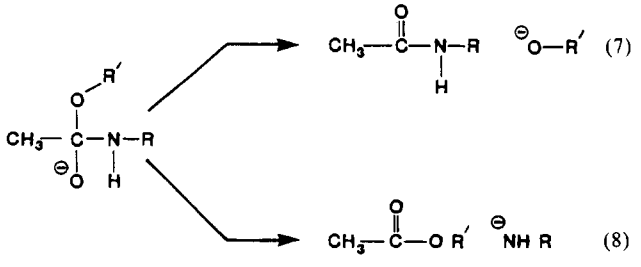
The ^{15}N evidence for a strong H-bond to $N^{\epsilon 2}$ is unequivocal. What is less certain, however, is the identity of the donor. Besides the hydroxyl group of Ser-195, however, there is only one other possible candidate for this role: water. The following arguments strongly support Ser-195 over water as the donor: First, the fact that the H-bond is broken by treatment of the enzyme with PMSF or DIFP supports Ser-195 over H_2O as the donor. Such treatment obviously would break a His–Ser H-bond, but is is not clear that it necessarily should also break a His–H_2O H-bond, even if the H_2O were an "ordered" H_2O molecule interacting with Ser-195. Second, the magnitude of the ^{15}N chemical shift change assignable to breaking the H-bond is quite large and thus indicative of a donor group with considerable proton-donating power. The hydroxyl group of Ser-195 (pK_a = 13.6) is a stronger acid than H_2O (pK_a = 15.7) by about 2 orders of magnitude (Bruice et al., 1962) and should thus be a correspondingly more powerful H-bond donor. Finally, the ^{15}N shifts of the model compounds listed in Table I represent nitrogen atoms surrounded by, and thus H-bonded to, H_2O because they were obtained in H_2O solutions. Thus, these ^{15}N shifts are in effect "standardized" relative to His–H_2O hydrogen bonding. Therefore, the 10 ppm downfield change in the shift of $N^{\epsilon 2}$ of His-57 from 138 to 128 ppm on reacting the enzyme with PMSF probably reflects not only the breaking of a His–Ser H-bond but also its replacement by a His–H_2O H-bond.

Ever since Blow's proposal for the operation of a "charge-relay", there has been considerable interest in determining the charge distribution across the Asp-His-Ser triad. The ^{15}N chemical shifts of His-57, because of their exquisite sensitivity to the H-bonding interactions with Asp-102 and Ser-195, offer a unique opportunity to address this question in a reliable and

quantitative way. A H-bond is often regarded simply as a proton at some intermediate stage of transfer from the donor atom to the acceptor atom. Assuming a linear relationship between the extent of proton transfer and ^{15}N chemical shift, a not unreasonable assumption in view of the demonstrated linear relationship between ^{15}N chemical shifts and N–H bond distances (R. G. Griffin, personal communication), and using 82 ppm (the difference between the ^{15}N shifts of type α and type β nitrogens) to represent 100% proton transfer, the 10 ppm *upfield* displacement of the ^{15}N shift of N^{ϵ_2} by the hydroxyl proton of Ser-195 indicates that this proton is about 12% along the way of being transferred to N^{ϵ_2}. Similarly, the ~10 ppm *downfield* displacement of the ^{15}N shift of N^{ϵ_1} of His-57 by the Asp–His H-bond indicates that the N^{δ_1}–H proton is also about 12% along the way of being transferred to the carboxylate of Asp-102. The above analysis suggests that the triad can be viewed as polarized with a charge distribution, in electrostatic units, of −0.88 on Asp-102, 0.0 on His-57, and −0.12 on Ser-195. However, this should not be interpreted to indicate that a serine alkoxide ion, **1b**, is actually formed as such in any amount in the resting enzyme. A H-bond is more correctly regarded as a single hybrid structure rather than as a mixture of two or more structures (Jencks, 1969). On this point it seems that Blow's proposal may have been widely misunderstood. In writing structures **1a** and **1b** of eq 1, he connected them with a double-headed arrow indicative of resonance structures, not of structures in equilibrium (Blow et al., 1969). As such, **1b** should be viewed as contributing to the actual structure of the triad but not as having independent existence in itself.

The Catalytic Mechanism. The present work, supporting a strongly H-bonded and polarized Asp-His-Ser triad, reinforces the idea that the Asp-His couple may play a role in enhancing the ability of the serine hydroxyl group to attack the carbonyl carbon of the substrate. Because the weight of experimental evidence still indicates that the immediate result of such attack is the formation of a tetrahedral intermediate (TI) and, moreover, because the uncatalyzed formation of such a species is likely to have a significant activation barrier, it seems reasonable to suppose that the triad is indeed designed to facilitate this reaction. The simplest mechanism is shown in eq 3. Here, the role of His-57 would be to provide essentially general base catalyzed assistance to Ser-195. The role of Asp-102 would be to increase the effectiveness of His-57 as a general-base catalyst by (i) ensuring the predominance of the N^{δ_1}–H tautomeric form of His-57, which puts the unshared pair of electrons on N^{ϵ_2} facing Ser-195, (ii) orienting the imidazole ring in the direction of Ser-195 and restricting its motion, and (iii) providing electrostatic stabilization of the incipient imidazolium ion.

However, this scheme fails to provide a fully satisfactory account of the catalytic power of serine proteases: A TI such as shown in **3b**, once formed, can decompose to regenerate starting materials by expelling the serine alkoxide ion (eq 7)



or to produce the acyl enzyme by expelling the amide ion (eq 8). In the absence of other factors, pathway 7 will be greatly favored over pathway 8 because the alkoxide ion (pK_b = 1.0) is a much better leaving group than the amide ion (pK_b = 16).

In the enzyme-catalyzed reaction, if it proceeds via the formation of **3b** as shown in eq 3, the situation would be even more unfavorable. This is because the Asp–His H-bond, which orients the imidazole ring of His-57 toward Ser-195 in the resting enzyme, in **3b** can only serve to keep the protonated imidazolium ion in a position to provide general acid catalyzed assistance to pathway 7, thereby increasing the already substantial advantage of this nonproductive reverse pathway over pathway 8, the productive forward pathway. Thus, although it could clearly facilitate the first step of the reaction, i.e., the formation of the TI, a strongly H-bonded Asp-His-Ser structure would actually inhibit the second and perhaps more important step, i.e., expulsion of the amide ion and cleavage of the C–N peptide bond. This was first pointed out by Polgar (1971) and would be true for a charge-relay mechanism as well as for a general-base mechanism.

This deficiency in our understanding of how the Asp-His-Ser triad functions in catalyses has been recognized as such by others and has been the basis for mechanistic conjecture. Jencks (Swatterthwait & Jencks, 1974) has proposed that His-57 may be moving or flipping rapidly between positions ideal for providing general base catalyzed assistance to the first step (i.e., formation of the TI) and for providing general acid catalyzed assistance to the second step (i.e., the breakdown of the TI to the acyl enzyme). Experimental evidence, however, favors a rigidly held histidine, at least in the resting enzyme. In addition, this scheme has been criticized on philosophical grounds as catalytically inefficient (Komiyama & Bender, 1979).

Wang (1970) has proposed that proton transfer may occur along bent hydrogen bonds. In this scheme, His-57 is rigidly held midway between the positions ideal for H-bonding to Ser-195 and to the nitrogen atom of the scissile peptide bond. Wang has argued that with this configuration the serine proton has a better chance of being transferred to the leaving group after its arrival on N^{ϵ_2} of His-57. Thus, His-57 in this scheme serves as a stationary proton relay.

More recently, Kraut has proposed that His-57 is rigidly held in position to form a strong H-bond with the nitrogen atom of the substrate and thus to provide catalytic assistance to the second step but not to the first (Kraut, 1977; Matthews et al., 1977). Kraut suggests that the driving force for the formation of the TI, the first step, comes from the ability of serine proteases to specifically bind and stabilize the TI.

The present finding that protonation of His-57 in PMSF- and DIFP-inactivated enzyme complexes triggers the disruption of the Asp–His H-bond suggests that serine proteases may have solved the above problem in a somewhat different way. Consider the seemingly contradictory nature of this H-bond disruption: Protonating His-57 in effect replaces a negative–neutral H-bonded pair with a negative–positive one. This change should strengthen the Asp–His interaction, not break it. Clearly other factors are at work.

Consider further that to be called into play these factors apparently require the presence of a tetrahedral adduct to Ser-195 in addition to a protonated imidazolium ion of His-57. (That neither a tetrahedral adduct to Ser-195 nor a protonated His-57 alone is sufficient to break the Asp–His H-bond is demonstrated by the clear presence of the Asp–His H-bond in the PMSF and DIFP complexes at high pH where His-57 is neutral and in the native enzyme at low pH where His-57 is protonated.) However, it is just this combination of a tetrahedral adduct to Ser-195 and a protonated imidazolium

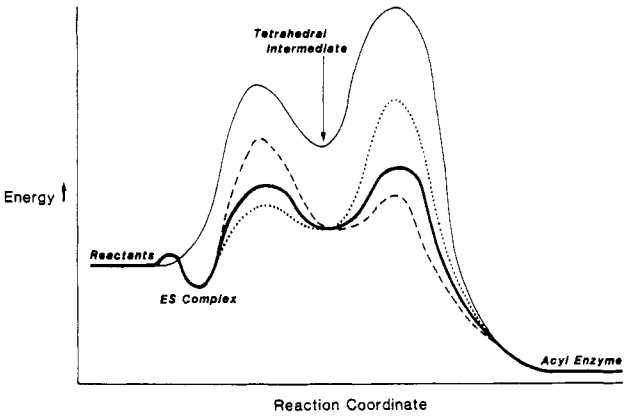


FIGURE 4: Hypothetical reaction profiles of various mechanisms: (—) uncatalyzed reaction; (···) stationary Asp–His couple hydrogen bonded to Ser-195; (– –) stationary Asp–His couple hydrogen bonded to nitrogen atom of scissile peptide bond; (—) moving histidine mechanism.

ion of His-57 that characterizes the putative TI structure **3b**, formed with real substrates.

These considerations suggest that the observed rupture of the Asp–His H-bond is a functional response, which, with real substrates, permits or directs the newly formed imidazolium ion of His-57 to move into a position to donate its proton to the nitrogen atom of the leaving group after the neutral form of His-57 has acquired the proton from Ser-195.

How is this Asp–His H-bond rupture accomplished? Because this H-bond is energetically favorable, and should become even more favorable as His-57 becomes protonated on forming the TI, its rupture must involve an interplay between the enzyme and substrate. One possibility is that the newly formed tetrahedral adduct to Ser-195 and protonated imidazolium ion cause a realignment of interactions, first between the enzyme and substrate and then within the enzyme itself, which more than compensates for the loss of the Asp–His H-bond. This conformational change may be global or may be localized near the active site. A second possibility is that there is insufficient space for His-57 to accept the serine hydroxyl proton without movement of the imidazole ring. From the X-ray structures, this seems less likely, but at present neither possibility can be ruled out.

Figure 4, by comparing the hypothetical energy requirements of the various mechanistic schemes, illustrates how such a moving proton shuttle mechanism might better explain the catalytic power of serine proteases than mechanisms involving a stationary histidine. The reaction profile for the conventional mechanism wherein His-57 remains fixed in a position ideal for interacting with Ser-195 clearly depicts the previously discussed deficiencies of this mechanism. In this scheme catalytic power is in effect squandered on interconverting reactants and the TI while the overall reaction, formation of the acyl enzyme, proceeds at a rate governed by a step, i.e., TI to acyl enzyme, which receives little or no catalytic assistance. The reaction profile for a scheme proposed by Kraut, wherein His-57 remains fixed in a position ideal for interacting with the nitrogen atom of the scissile bond, shows that it has the reverse problem; i.e., the first step receives little or no catalyic assistance while catalytic power is squandered on the second. The reaction profile for the mechanism proposed here, wherein the Asp–His H-bond breaks on forming the TI state, shows that while this scheme would not be as effective as the conventional mechanism in lowering the first energy barrier, nor as effective as Kraut's scheme in lowering the second energy barrier, overall it might be the most effective in accelerating the rate of the formation of acyl enzyme.

How would the energy requirements for Wang's scheme of proton transfer along bent H-bonds compare with those of the above-described moving histidine mechanism? To a first approximation one might expect them to be very similar; i.e., both energy barriers would be lowered relative to the nonenzymatic reaction but not by as much as they would be if proton transfer moved along linear H-bonds. Which mechanistic scheme is capable of providing the greatest rate acceleration is a matter for conjecture. However, the moving histidine mechanism proposed here at least has some experimental support.

The mechanism proposed here at least provides a working model for the catalytic mechanism, which can be subjected to further testing: If the presently observed rupture of the Asp–His H-bond is a functionally significant response, then it should also be a general one; i.e., other serine proteases inhibited by PMSF or DIFP should exhibit this response on lowering the pH, and other inhibitors that form tetrahedral adducts to Ser-195 with the correct stereoelectronic characteristics should be able to substitute for PMSF or DIFP.

There are some reports in the literature that suggest that this phenomenon may indeed be a general one. Perhaps the most significant of these comes from X-ray diffraction studies of *Streptomyces griseus* protease A (Brayer et al., 1979b; James et al., 1980). (*S. griseus* protease A is a microbial serine protease that has extensive three-dimensional homology with the pancreatic serine proteases but relatively little sequence homology.) In this study, binding of a tetrapeptide aldehyde to the enzyme at pH 4.1 produced "a very large conformational movement of the imidazole ring of His-57", while binding of the corresponding tetrapeptide alcohol did not. Aldehydes, but not alcohols, form covalent tetrahedral hemiacetal adducts with Ser-195. This result shows that the combination of a protonated His-57 and a tetrahedral adduct to Ser-195 is again necessary and sufficient to trigger a major conformational movement of His-57, which includes the rupture of the Asp–His H-bond, in an enzyme–inhibitor complex where both the enzyme and the inhibitor are different from the ones studied here. This study attributed the movement of His-57 to prohibitive close contacts between the tetrahedral hemacetal adduct formed with Ser-195 and the imidazole ring of His-57 in the native position.

In a study of another very similar complex formed between SPGA and chymostatin, a naturally occurring peptide aldehyde, the X-ray diffraction data indicate that His-57 does not become displaced (Delbaere & Brayer, 1985). At first, this result may seem to be at odds with the present hypothesis. However, the X-ray data also indicate that complexation of chymostatin to SPGA at low pH increases the mean thermal displacement of His-57, with the largest increase centered at $N^{\delta 1}$ of His-57. This result suggests that the Asp–His H-bond in this complex must be at least weakened if not broken. Nevertheless, the observation of increased freedom of motion for His-57 in itself supports the present hypothesis. In addition, the authors of the chymostatin–SPGA complex X-ray study noted that His-57 is sterically prevented from occupying the position it occupies in the synthetic peptide aldehyde complex by the P2 leucyl side chain of chymostatin.

Another experimental result that supports the idea that this phenomenon may be a general one comes from a ^{13}C NMR study of a complex between α-lytic protease and a tripeptide aldehyde (Hunkapiller et al., 1975), which demonstrated that the protonation of His-57 at low pH in the aldehyde–enzyme complex conferred increased mobility on His-57. The complex

with the corresponding tripeptide alcohol failed to show this effect. These results again are consistent with the hypothesis that the combination of a tetrahedral adduct to Ser-195 and a protonated His-57 triggers the rupture of the Asp–His H-bond.

Conclusions

The ^{15}N NMR chemical shifts reported here demonstrate that a rather strong H-bond links His-57 and Ser-195 of the catalytic triad of α-lytic protease. In addition, the ^{15}N chemical shifts support Blow's original discription of the Asp-His-Ser triad as a strongly H-bonded and partially polarized system. However, this should not be interpreted as support for the existence of a serine alkoxide ion in the resting enzyme. Finally, the ^{15}N results also demonstrate that protonation of His-57 at low pH in PMSF- and DIFP-inactivated enzyme complexes triggers the rupture of the Asp–His H-bond. The similarity of the PMSF- and DIFP-inhibited enzymes at low pH to the putative tetrahedral intermediate indicates that this rupture represents a functional response.

These results reinforce the idea that the Asp–His couple plays a role in activating the serine hydroxyl group for nucleophilic attack on substrate. In addition, they indicate that the catalytic mechanism of serine proteases involves an interplay between enzyme and substrate such that the imidazole ring of His-57 moves from a position designed to activated Ser-195 and facilitate the formation of the TI to a position designed to facilitate the breakdown of the TI to the acyl enzyme during the course of the reaction.

Registry No. EC 3.4.21.12, 37288-76-9; L-His, 71-00-1; serine proteinase, 37259-58-8; imidazole, 288-32-4; $N^{\delta 1}$-methylimidazole, 616-47-7.

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