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Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy. The State of Histidine in the Catalytic Triad of α-Lytic Protease. Implications for the Charge-Relay Mechanism of Peptide-Bond Cleavage by Serine Proteases[1,2]

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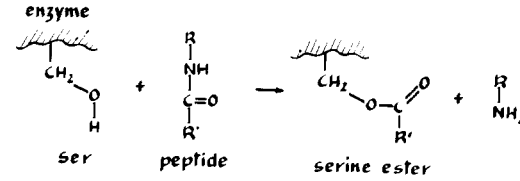
Abstract: Histidine, enriched in ^{15}N in the imidazole ring, has been incorporated into the "catalytic triad" of serinyl, histidyl, and aspartyl residues of α-lytic protease, using a histidine auxotroph of myxobacter 495. The pK_a of this histidyl residue is 7.0 ± 0.1 at 26 °C, as determined by the changes of its ^{15}N chemical shifts in nuclear magnetic resonance spectra. This finding is contrary to previously published reports that histidyl residues at the active sites of serine proteases are likely to be abnormally weak bases, while the "buried" aspartyl residues of the catalytic triads are likely to be abnormally weak acids and thus offers no support for the current formulation of the charge-relay mechanism of action of serine proteases. The ^{15}N chemical shifts further demonstrate that, at catalytically active pH values, the tautomer with hydrogen on N3 (the π nitrogen) of the imidazole ring predominates. This is an unusual tautomeric state both for simple 4-substituted imidazole derivatives and for histidyl residues in proteins, and its predominance in α-lytic protease can be reasonably attributed to a hydrogen-bonded interaction between NH at the 3 position and the adjacent "buried" carboxylate group of aspartic acid. The implication of the results for the respective roles of the aspartyl and histidyl residues in serine protease catalysis is discussed in terms of the energy requirements of the individual steps for the formation and breakdown of the serine ester intermediate.

Hydrolytic cleavage of peptide bonds, such as amino acids linked together in proteins, is an energetically favorable reaction, but one that normally takes place very slowly at room temperature, even in the presence of rather strong acids or bases. Hydrolysis, however, can be strongly catalyzed by many enzymes, and much effort has been expended to determine how these proteases have the ability to increase the rate of hydrolysis of peptide bonds in other proteins by a millionfold or more in neutral solutions. Proteases are of several general types and one of these, the *serine protease* family, is characterized by the presence at the active site of a "catalytic triad" comprised of the side-chain residues of serine, histidine, and aspartic acid.

First discovered through X-ray diffraction studies of chymotrypsin,[3-5] the presence of the triad has been similarly demonstrated for trypsin,[6] elastase,[7,8] *S. griseus* protease B,[9] and α-lytic protease[10]—all of which are structurally homologous. The triad is also present in subtilisin,[11,12] a bacterial protease quite dissimilar to the chymotrypsin proteases in other respects and therefore a possible example of convergent evolution at the molecular level.

A key feature of the mechanism of action of the serine proteases is an attack by the hydroxyl group of the serine located at the active site on the carbonyl group of the peptide at the cleavage point, forming an ester and liberating an amino group, eq 1.[13] Because uncatalyzed alcoholysis of peptide



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bonds, like hydrolysis of such bonds, is thermodynamically favorable, but kinetically very slow, the fact that the ester is formed is not itself helpful in understanding why the enzymatic hydrolysis is so facile.

The vital role of histidine in catalysis by serine proteases is well established[14] and, because the activity of these proteases decreases with increasing acidity to just the degree that the imidazole ring of the histidine at the active site would be expected to become converted to its conjugate acid, the unionized imidazole ring has been implicated as the active form of the histidine. Blow's discovery[5] for α-chymotrypsin that Asp 102, His 57, and Ser 195 seem nicely aligned to act in some concerted fashion in peptide hydrolysis led to the proposal of a "charge-relay" mechanism which was subsequently modified by Richards and co-workers[15] to a sequence wherein attack of the serine hydroxyl oxygen on peptide complexed with the enzyme (the "enzyme–substrate complex") occurs simultaneously with removal of the serine hydroxyl hydrogen by histidine at N1 (the τ nitrogen)[16] and transfer of a proton from

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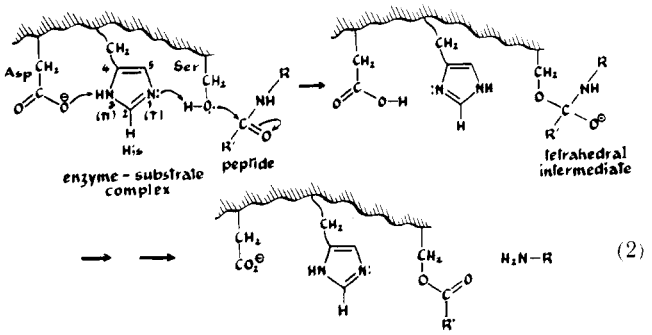
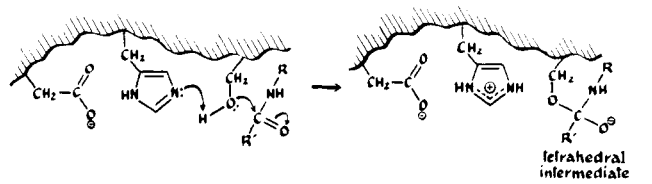
N3 (the π nitrogen) to the carboxylate oxygen of the aspartyl residue (eq 2). The overall result of this process is formation



(2)

of the so-called "tetrahedral intermediate" which decomposes to the serine ester with cleavage of the peptide bond.

If the charge-relay mechanism is to account for the catalytic activity of the enzyme, it is necessary that it be energetically reasonable, at least to the degree that the postulated products need to be more stable than those that would be formed without transfer of a proton to the aspartyl carboxylate anion.[17] Unless being incorporated into an enzyme drastically changes the chemical character of the groups involved, eq 3 is expected to



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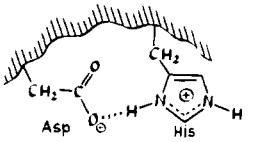
be more favorable than the first step of eq 2 by somewhat more than 3 kcal/mol, because imidazolium cations ($pK_a \sim 7$) are normally weaker acids than the terminal carboxyl of aspartic acid ($pK_a \sim 4.5$).

This difficulty with the charge-relay mechanism has been addressed by Richards and co-workers[15] who prepared α-lytic protease (a member of the serine protease family) enriched in ^{13}C at the 2 position of its single histidine. They reported that the NMR spectrum of the enriched enzyme indicated that the histidine does not become protonated until the pH is reduced to below 4. The corollary of this is that the free aspartyl carboxyl, located in a hydrophobic pocket of the enzyme, is the group having the pK_a of 6.7, and that it produces inactive enzyme when protonated. The crux of the Richards work is that the structure of the enzyme in the neighborhood of the catalytic triad is such as to make the histidine imidazole ring a weaker base by three orders of magnitude and the aspartyl carboxyl a weaker acid by two orders of magnitude. These results favor formation of the tetrahedral intermediate by the route of eq 2 rather than eq 3.

Corroboration of the postulated weakness of the aspartic acid carboxyl in the catalytic triad has been provided by Koeppe and Stroud[18] who report an infrared absorption of trypsin, which corresponds to an acid of pK_a 6.8, and which they assign to the appropriate aspartyl carboxyl. Further support has been supplied by study of model systems[19] and theoretical calculations.[20] These results, however, cannot be described as settling the issue of whether the charge-relay mechanism is important to understanding the catalytic effectiveness of serine proteases for hydrolysis of peptide bonds.[21] Thus, Robillard and Shulman[22] report very low-field ^1H NMR resonances for chymotrypsin, trypsin, subtilisin, and α-lytic protease, which could be assigned to protons in hydrogen bonds between the histidyl and aspartyl residues of the catalytic triads of these enzymes. The pH dependences of these resonances correspond to acids of $pK_a \sim 7.5$ and were concluded to arise from imidazolium cations hydrogen bonded to aspartyl carboxylate anions, **1**. Further uncertainty comes from the work



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of Markley and Porubcan[23] who report that the NMR resonance of the proton on C2 of the imidazole ring of the histidyl residue of the catalytic triad in porcine trypsin changes with pH in such a manner as to correspond to an acid of $pK_a = 4.5$ and thus is intermediate in strength between the values obtained by Richards and co-workers[15] and Robillard and Shulman.[22]

In the present work, we have studied the ionization behavior of the histidine of the catalytic triad of α-lytic protease using ^{15}N NMR spectroscopy. This technique is expected to be especially informative about the state of protonation, hydrogen-bond formation, and tautomeric equilibrium of imidazole rings on the basis of the nitrogen NMR studies of Rüterjans and co-workers[24] with ^{15}N-enriched histidine, and ^{14}N results obtained by Witanowski and co-workers[25] on imidazoles. It seemed especially appropriate to investigate α-lytic protease because of its similarity to the mammalian serine proteases and the extensive studies already made of its ionization behavior. Furthermore, the work of Richards and co-workers[15] offered the certainty of being able to enrich the nitrogens of this enzyme's single histidine to facilitate taking ^{15}N NMR spectra. This last is important because, while it is conceivable that the desired spectra might be obtained at the natural-abundance level of ^{15}N, very large quantities of material and undesirably high enzyme concentrations would be required.[26] To achieve efficient and specific incorporation of ^{15}N-labeled histidine into α-lytic protease, we have induced and isolated an auxotroph of myxobacter 495 for which histidine is an essential amino acid. With the aid of this mutant, it has been possible to obtain substantial quantities of ^{15}N-enriched α-lytic protease with relatively small amounts of labeled histidine in the growth medium. The behavior of the ^{15}N NMR resonances of this labeled α-lytic protease as a function of pH is the subject of this paper.

Experimental Section

Materials. L-Histidine, selectively enriched with ^{15}N (99%) at the 3-(π)-nitrogen of the imidazole ring was obtained from Isotope Labelling Corporation. D,L-Histidine enriched in ^{15}N to the extent of about 95% at both imidazole nitrogens was supplied by Rohstoff-Einfuhr, GmbH. The Ac-L-Ala-L-Pro-L-Ala 4-nitroanilide used to assay the activity of the enzyme was generously provided by Dr. M. W. Hunkapiller and Professor J. H. Richards.

Growth of the Auxotroph of Myxobacter 495. The procedure for induction, isolation, and characterization of the mutant of myxobacter 495 used to maximize the formation of α-lytic protease containing ^{15}N-labeled histidine will be described elsewhere. An absolute requirement for histidine was demonstrated for this mutant. D-Histidine could be substituted for L-histidine, but when this was done, near normal growth rates were observed only after a substantial induction period. The α-lytic protease produced by the mutant was fully active toward Ac-L-Ala-L-Pro-L-Ala 4-nitroanilide and otherwise appeared identical to the enzyme produced by wild-type myxobacter 495.

The procedures described by Richards and co-workers[15] and Whitaker[27] for culturing myxobacter 495 were modified to minimize dilution of the isotopically labeled histidine by the ordinary histidine in the casein hydrolysate. The casein amino acids were replaced by an amino acid mixture designed to simulate casein less histidine. In general, 2 g/L of this amino acid mixture was employed in the liquid culture medium along with 12 g/L of monosodium glutamate and 10 g/L of sucrose. The inorganic salt content was $K_2HPO_4 \cdot 3H_2O$, 1.2

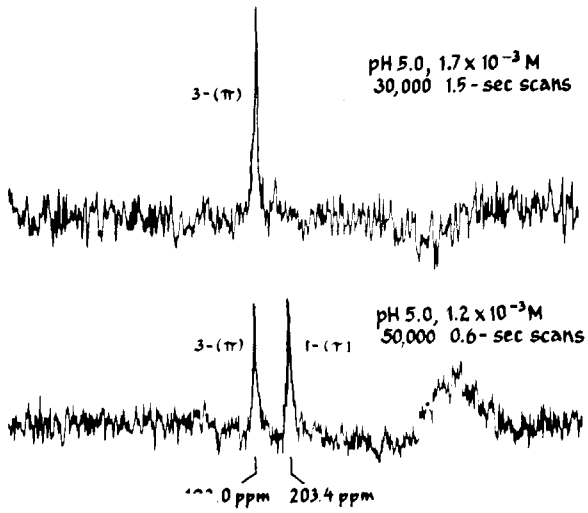


Figure 1. Proton-coupled ^{15}N NMR spectra, at 18.2 MHz, of α-lytic protease from an auxotroph of myxobacter 495 + ^{15}N-labeled histidine (1 His/molecule, mol wt 19 860).

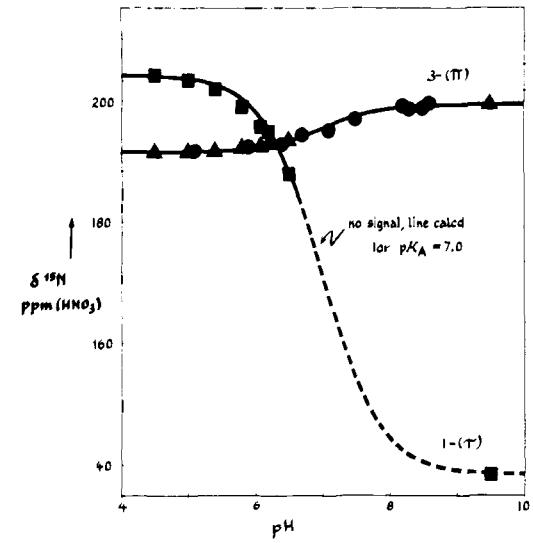


Figure 2. Dependence of ^{15}N shifts of ^{15}N-enriched histidine nitrogens in α-lytic protease as a function of pH: ●, ^{15}N enriched at N3; ■ and ▲, enriched at N1 and N3, respectively.

g/L; NaCl, 1.2 g/L; $MgSO_4 \cdot 7H_2O$, 0.6 g/L; $Fe_2(SO_4)_3$, 15 mg/L; $ZnSO_4 \cdot 7H_2O$, 4 mg/L, and $MnSO_4 \cdot H_2O$, 3 mg/L.

The culture was maintained on agar slopes (1% agar, 0.2% tryptone) and regularly checked for the appearance of back mutants and/or contamination with histidine-producing organisms by replica streaking onto separate plates lacking histidine and containing 20 mg/L of histidine. These plates were 1.5% agar and also contained 2 g/L of glutamate, 1 g/L sucrose, and one-fifth the inorganic salt content described for the liquid culture medium.

Incorporation of ^{15}N-Enriched Histidine into α-Lytic Protease. Single colonies of the mutant were isolated and a requirement for histidine verified for each. These colonies were used to inoculate slopes which were then incubated for 36–48 h and, after transfer, used in turn to inoculate 50-mL liquid-shake cultures made up as described above, but also containing 100 mg/L of labeled histidine. The 50-mL cultures were shaken at 30 °C for 36–48 h and used as inocula for 0.5-L liquid-culture media contained in 2.8-L Fernbach flasks and supplemented with 20–80 mg/L of labeled histidine. The optimum yield of α-lytic protease (in terms of efficient use of labeled histidine) was about 90 mg/L with a starting labeled histidine concentration of 60 mg/L. Prior to collecting the supernatant for isolation of the enriched α-lytic protease, each culture was checked for back mutation or contamination as described above, but in no case were cells not demonstrating a requirement for histidine detected.

The α-lytic protease was isolated as previously described,[15] except that a continuous gradient was used in place of a stepwise gradient. Both the singly and doubly labeled enzyme preparations were fully active and the enriched samples appeared homogeneous on polyacrylamide gel electrophoresis in basic buffer.

Sample Preparation. The α-lytic protease was exhaustively dialyzed against 6 mM EDTA solutions in doubly distilled water, followed by dialysis against doubly distilled water prior to lyophilization. All glassware used for preparing the NMR samples was thoroughly rinsed with double distilled water. The α-lytic protease was dissolved in 0.1 M KCl and transferred to a 25-mm tube. The pH of the sample was measured directly in the NMR tube, using a Radiometer Model PM pM meter, and recorded both before and after each spectrum. The pH was adjusted using 1.0 N HCl or 1.0 N NaOH solutions made from "ultra-pure" materials supplied by Alfa Products.

About a 20% decrease in enzyme activity as measured by the rate of hydrolysis of Ac-L-Ala-L-Pro-L-Ala 4-nitroanilide occurred during the 5 days required to take the spectra over the range of pH values.

^{15}N NMR spectra were obtained with the previously described Bruker WH-180 spectrometer[26] at 18.25 MHz in the pulsed Fourier-transform mode. A concentric 5-mm tube containing 0.1 M solution of ^{15}N-enriched nitric acid in D_2O was used as the ^{15}N reference and deuterium field-frequency lock signal. The spectra normally had a sweep width of 7000 Hz, a 90° pulse angle (70 μs), and an acquisition time of 0.58 s. Usually, proton decoupling was not employed, and the sample temperature was near 26 °C.

Results

^{15}N NMR Spectra of 3-(π)-^{15}N-Labeled Histidyl α-Lytic Protease. A representative ^{15}N NMR spectrum of α-lytic protease obtained from 3-(π)-^{15}N-labeled histidine is shown in Figure 1. Other than the broad, relatively weak resonances at 245–265 ppm arising from the amide nitrogens in the peptide backbone of the enzyme, only a single resonance is observed, which can be assigned to the 3-(π)-nitrogen of the histidine of the catalytic triad. The pH dependence of the position of this resonance is shown in Figure 2, and it will be seen that there is an upfield chemical shift with increasing pH from 191.6 ppm at pH 4.5 to 199.4 ppm at pH 8.5. The shape of the pH curve is consonant with titration of an acid with a pK_a of 7.0. With proton decoupling, the line width of this resonance at pH 5.1 is ~13 Hz, while without proton decoupling, the line width is ~20 Hz. When the pH is increased, both line widths increase and are 55 Hz (proton decoupled) and 70 Hz (no proton decoupling) at pH 8.3. As with other imidazoles in aqueous solution[28] and with histidine itself,[24] N–H exchange with the solvent is fast and no direct ^{15}N–H couplings (which should be 60–90 Hz) were observed. Two- and three-bond couplings from N3 to the ring CH hydrogens would be expected to contribute at most ~7 Hz to the line width at pH 5.1 and ~15 Hz at pH 8.3.[24] The results of measurement of T_1 (0.25 ± 0.1 s) and of the proton–nitrogen nuclear Overhauser effect (0.8 ± 0.1) at pH 5.1 are consistent with expectations for a system of this type.

^{15}N NMR Spectra of 1,3-(τ,π)-^{15}N-Labeled Histidyl α-Lytic Protease. The nitrogen NMR spectrum of the α-lytic protease prepared from doubly labeled histidine shows two ^{15}N resonances (Figure 1). One of these behaves identically with variation of pH as for the singly labeled enzyme, and this is clearly the 3-(π)-nitrogen of the imidazole ring (Figure 2).

In contrast to the rather small (7.8 ppm) *upfield* chemical-shift change observed for N3 on going from pH 4.5 to 8.3, N1(τ) exhibits a large (66 ppm) *downfield* shift change in going from 204.2 ppm at pH 4.5 to 138 ppm at pH 9.5. Again, the histidine residue is clearly acting as an acid with a pK_a of about 7. The positions of these resonances are fully reversible over the pH range investigated.

The line widths of the N1(τ) and N3(π) resonances are comparable at both low and high pH values and no clear evidence for one-bond ^{15}N–H couplings was obtained. A serious problem was encountered with the N1 resonances over the pH range 6.5–9.0 when the signal strength simply decreased below

Table I. ^{15}N Chemical Shifts of Some Imidazole Derivatives in Acid and Neutral Aqueous Solutions at 25 °C in ppm Relative to $D^{15}NO_3$[a]

compd		imidazolium cation[b]	imidazole[c]	Δ[d]	pK_a	av shift of imidazolium cation[e]	av shift of imidazole[f]
imidazole		202.0	171.0	31.0	6.95	202.0	171.0
1-methylimidazole	N1	204.1	211.5	−7.4		203.9	169.9
	N3	203.6	128.5	75.1			
4-methylimidazole	N1	202.6	172.8	29.8	7.5	200.6	168.6
	N3	198.6	164.4	34.2			
imidazole-4-acetic acid	N1	203.0	180.0	23.0	7.35	201.4	167.8
	N3	199.7	155.6	44.1			
histidine[g]	N1	201.5	196.5	5.0	6.2	200.3	169.8
	N3	199.0	143.1	55.9			
	N1		180.8	20.7			168.9
	N3		157.0	42.0			
α-lytic protease	N1	204.2	138.0	66.2	7.0	197.9	168.7
	N3	191.6	199.4	−7.8			

[a] Positive shifts are upfield. [b] Shifts under conditions of full protonation. [c] Shifts under conditions of no protonation. [d] Changes in shift from neutral to cationic imidazole ring. [e] Average shift of nitrogens in imidazolium cation. [f] Average shift of nitrogens in neutral imidazole ring. [g] Shift data from Rüterjans and co-workers[24] recalculated to $D^{15}NO_3$ standard. One set of data for neutral imidazole is for histidine amphion and the other for the histidine anion.

limits of detection. This kind of behavior could be due to intermediate exchange rates in which chemical shift or coupling differences are not averaged, paramagnetic ion induced relaxation of the N1 resonance occurs, or some other extreme line-broadening influence associated with shortening T_2 and/or T_1 of this nitrogen is observed. Intermediate exchange rates seem unlikely to be the cause, because they would be expected also to influence the N3(π) resonance, at least to some degree comparable with their effect on the N1 resonance.

Paramagnetic ions are especially likely to be the source of the loss of signal intensity at the intermediate pH values for the following reasons: (1) although many precautions were taken to eliminate contamination by heavy-metal ions, atomic absorption analyses of an aliquot of the solution used for the NMR spectra indicated the presence of 1.9 ppm of iron and 0.17 ppm of copper; (2) a decrease in the line width of the ^{15}N resonance at higher pH values is expected because either the 0.3 mM EDTA added to these solutions or hydroxide ions compete for binding of the metal ions;[29] (3) a sharp decrease in the T_1 of N1(τ) in ^{15}N-enriched histidine over the range pH 5.0–8.0 has been reported[24] and we have found the N3 resonance of 1-methylimidazole to be broadened to near the limit of detectability at pH 6.6; (4) as will be shown later, most of the N1(τ) nitrogens of α-lytic protease do not carry a proton in neutral solutions and are thus expected to complex more efficiently with metal ions than the N3(π) nitrogens; (5) heavy-metal ions are known to form complexes involving Asp and His at the catalytic site of trypsin.[18,30]

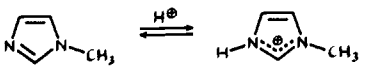
In any case, the ^{15}N chemical shifts that could be measured for N1(τ) over the range of pH 4.5 and 9.5 serve at least as boundary values for estimating the pK_a of histidine of α-lytic protease. Thus, pK_a = 7.0, which fits the pH dependence of the resonance of N3 in the enzyme, can be seen to give an excellent fit to the available data for N1(τ) as well (Figure 2). We conclude therefore that the histidine of α-lytic protease behaves as a normal imidazole with respect to basicity. We turn next to the problem of using the NMR data to extract as much structural information as possible about the condition of the histidine of α-lytic protease at different pH values.

Comparison with Model Compounds. A characteristic of imidazoles with an N–H bond is that they exist as an equilibrating mixture of tautomeric forms, eq 4. These equilibria are rapidly established in water solution at room temperature, with rate constants >500 s^{-1}. The equilibrium constants K of eq 4 depend on the nature of R but obviously for imidazole itself K

(4)

will be unity, and because equilibration is fast, the nitrogen NMR resonances appear to have the same chemical shift which for imidazole is 171 ppm (Table I).

However, it is well known from a variety of studies that the >NH and ≫N nitrogens of imidazole should have a very large difference in chemical shift.[24,25,31] 1-Methylimidazole (**2a**) provides an excellent model to demonstrate this, because substitution of methyl for hydrogen on nitrogen in piperidines produces at most only a small ^{15}N-shift difference for the nitrogen,[28] and because equilibration of the type represented by eq 4 does not occur at a measurable rate in water at room temperature. The difference in ^{15}N shift between the nitrogens of **2a** is 83.3 ppm, and we have to expect that there would be nearly the same difference in shift between the nitrogens of imidazole if equilibration by eq 4 were slow. That **2a** is a reasonable model for the shifts of the individual tautomers is supported by the fact that the average of the shifts of **2a**, 169.9 ppm, is only 1.1 ppm different from the observed average shift of imidazole itself.



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2a **2b**

On protonation of imidazole, the average position of the nitrogen resonances moves upfield by 31 ppm. The nitrogens are equivalent in the imidazolium cation **3** so the problem posed by the tautomeric equilibrium of eq 4 no longer applies. Again, 1-methylimidazole seems to behave analogously to imidazole in that the nitrogen shifts of its conjugate acid **2b**, formed as in eq 5, differ by only 0.5 ppm and their average is less than 2 ppm different from the nitrogen shifts of **3**.

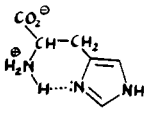
3

We need next to decide whether substitution of a saturated group at the 4 position will cause such fundamental changes in the ring system (in addition to making K of eq 4 $\neq 1$) as to vitiate comparisons between such substances and other imidazoles. The data of Table I can only be used as an argument for suggesting that, other than possible effects on K, the in-

fluence of a saturated 4 substituent on the ^{15}N shifts represents at most a rather small perturbation. Thus, other than for α-lytic protease, the average shifts of the nitrogens of the 4-substituted imidazolium cations differ from those of imidazole or 1-methylimidazole by less than 4 ppm, and the average shifts of the corresponding neutral forms differ from those of their counterparts by less than 3.3 ppm.

The magnitude of the K of eq 4 is of special interest for α-lytic protease because it could give information about the surroundings of the histidyl residue of the enzyme. It would be quite inappropriate to use histidine itself as a model for what one should expect for a nonterminal histidyl residue in a peptide chain because K for histidine is greatly affected by hydrogen-bonding interactions of N3(π) of the imidazole ring with the α-ammonium group.[24]

When the imidazole ring of the histidine in α-lytic protease is not protonated, it has what appears to be quite normal, average nitrogen shifts (Table I). However, K of eq 4 is clearly *greater* than 1, because the N3(π) shift is upfield of the N1(τ) shift in contrast to free histidine where the opposite is true.[24] Furthermore, K is less than 1 for 4-methylimidazole,[32] and this indicates that, for α-lytic protease, there is some interaction perturbing the tautomeric equilibrium in a manner analogous to (but opposite in direction to) the way that the ammonium group of free histidine interacts with the N3 nitrogen (**4**) at pH



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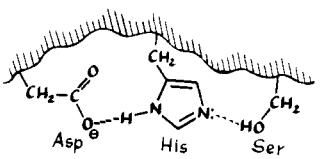

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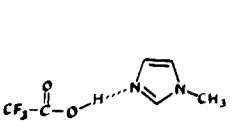
8 to cause K to be very much less than 1.[24] Such an interaction is possible with the carboxylate anion of the Asp residue making up the catalytic triad **5** and may be reinforced by further hydrogen bonding of N1(τ) to serine as in the formulation given by Robillard and Shulman.[22] Judging from the shifts of 1-methylimidazole, K appears to be about 6 for the imidazole ring in α-lytic protease, which corresponds to stabilization by the interactions represented by **5** of about 1.1 kcal/mol. It is significant that other studies of histidyl residues in peptides,[33] thyrotropin-releasing factor,[34] and myoglobins[35] show that $K < 1$ for these substances.

At low pH, the average ^{15}N shifts of the imidazolium cation of α-lytic protease will be seen from Table I to be about 2.8 ppm more toward lower field than for the cations of the other 4-substituted imidazoles studied. Furthermore, the chemical-shift difference between N1 and N3 is about 12.6 ppm compared to an average of 3.5 ppm for the other 4-substituted imidazolium cations. This behavior has a close parallel to that observed for 1-methylimidazole in chloroform solution in the presence of an equivalent of trifluoroacetic acid. In this solution, the average ^{15}N shift is 6 ppm less than that of the protonated form in water, and the difference between the N1 and N3 shifts is 16 ppm.[32] The fact that the average shifts are less than normal suggests that there is some (probably small) degree of charge transfer from the imidazolium cations to a neighboring carboxylate anion as the result of hydrogen bonding, **6** and **1**. The electrical asymmetry produced by the

6

hydrogen bond then accounts for the differences in ^{15}N shifts between N1 and N3. It may be questioned whether trifluoroacetic acid in chloroform is the equivalent of the free carboxyl of an aspartyl residue in a hydrophobic region of an enzyme, but there can be no question that the pattern of shifts is very similar.

One might also argue that the unusual shifts ascribed to **6** and **1**, in fact, arise from the alternative structures **7** and **8**,



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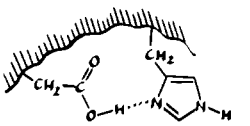


8

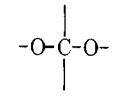
respectively. Hydrogen bonding does indeed result in changes in ^{15}N shifts which are in the same direction as protonation shifts, but are much smaller. Thus, the N1 and N3 resonances of 1-methylimidazole come at 215.7 and 111.4 ppm, respectively, in cyclohexane solution[36] and shift to 214.2 and 128.0 ppm when made up to 2 M in chloroform containing 2 M trifluoroethanol.[32] When 2 M acetic acid is substituted for the trifluoroethanol, the shifts change to 213.2 and 143.3 ppm as expected for enhanced hydrogen bonding,[32] but these shifts are far from the 191.6 and 204.2 ppm figures for the corresponding nitrogens in α-lytic protease. When protonation is facilitated in the acetic acid–chloroform solutions of 1-methylimidazole by adding a small amount of water, the shifts change to 198 and 203 ppm as expected for formation of **2b** (Table I).

Discussion

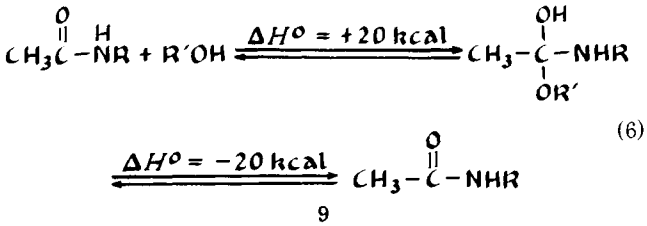
Bearing of the Present Results on the Charge-Relay Mechanism. If the charge-relay mechanism for serine proteases requires that the pK_a of the Asp residue in the catalytic triad be comparable to or larger than the pK_a of the conjugate acid of the histidine of α-lytic protease,[15,17] then the changes in the ^{15}N chemical shifts of this histidine with pH are most simply regarded as eliminating this mechanism as an important element in explaining why the catalytic triad is so effective. The original proposal,[5] that N1 of the histidine activates the serine hydroxyl through hydrogen bonding, is no longer supported by X-ray diffraction studies of subtilisin and other serine proteases,[37] including α-lytic protease.[10] However, the alignment of the groups in the pancreatic trypsin inhibitor–trypsin[38] and soybean trypsin inhibitor–trypsin complexes[39] is very favorable for removal of the serine hydroxyl by the histidyl residue to form the tetrahedral intermediate.

What then is the role of the His-Asp couple of the triad? The view we take here of the mechanism is not especially original and is surely oversimplified because it ignores the many possible secondary hydrogen-bonding and van der Waal's interactions between enzyme and substrate. Be that as it may, we feel it is desirable to take a somewhat different tack than most current discussions [except those of Lipscomb and co-workers[20]] through focusing on the energetics of formation and decomposition of reasonable models for the tetrahedral intermediate and how the His-Asp couple could facilitate these processes.

From published thermochemical data,[40] bond energies,[41] and the mean of about 5 kcal derived by Pople and co-workers[42] for the stabilization energy of the structural unit



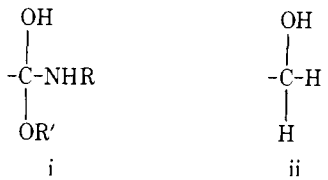
one can estimate that addition of ethanol to *N*-butylacetamide in the gas phase to form a tetrahedral intermediate **9** has $\Delta H^\circ \sim 20$ kcal, and decomposition to ethyl acetate plus butanamine has ΔH° ca. -20 kcal, eq 6 ($R = C_4H_9$, $R' = C_2H_5$).



(6)

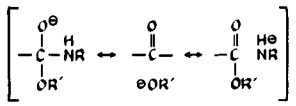
9

Neglecting possible differences in solvation energies of the participants, the extent to which ΔH° for formation of **9** would become more favorable in solution with alkoxide ion in place of an alcohol depends on the difference in acidity between **9** and the alcohol. This difference is difficult to evaluate, because little information is available to decide on how great an increase in acidity one should expect for the OH of the structural entity i as compared to ii. However,



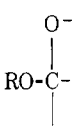
i ii

we do know methoxyacetic acid is a 20-times stronger acid than acetic acid, and the alkoxy group in this acid is one bond farther away from the acidic proton than in **9**. Furthermore, there is no possibility of hyperconjugative stabilization of the methoxyacetate anion by resonance interactions of the type exemplified by **10**. If the conjugate bases of entities like **9** were,



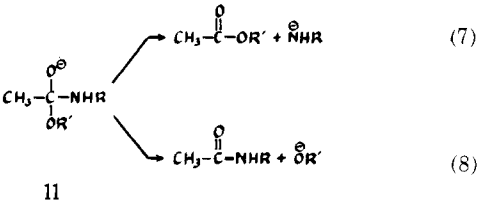
10

in fact, sufficiently stabilized by induction and resonance so that their pK_a's would approach 7, then, in the enzymatic reactions, the His-Asp couple could certainly provide not only stabilization of the transition state for attack of serine, as in eq 3, but, after accepting the serine hydrogen as a proton, might not be so strongly acidic to convert the conjugate base of **9** essentially completely to **9**. This combination of events requires that a tetrahedral intermediate corresponding to **9** would have to be a 10^5–10^6-times stronger acid than the serine alcoholic hydroxyl which has a pK_a of about 13.[43] This difference amounts to a free-energy difference of 7–8 kcal at room temperature and may be unreasonably large but, at least, it has the prospect of being subject to experimental test. An intriguing possibility suggested by Pople's theoretical calculations[42] is that the stereochemistry of the tetrahedral intermediate is fixed by the structure of the enzyme to take full advantage of the very large potential stabilization (>11 kcal)



if the rotational angle around its RO–C bond were to have the optimum value.[44]

Cleavage of the tetrahedral intermediate **9** to amine plus ester and reversion to amide plus alcohol (eq 6) were estimated above to have essentially equal ΔH values. However, the decomposition reactions of the conjugate base of **9** (**11**) to amide plus alkoxide or ester plus amide anions are surely not equally favorable (eq 7 and 8). Indeed, the difference in pK_a between



(7)

(8)

11

amines ionizing to RNH^- and alcohols to RO^- is $\sim 10^{15}$, which corresponds to 20 kcal and suggests that eq 7 may not even be energetically favorable. Consequently, a powerful acid catalysis is expected for decomposition of **11** to ester and amine. Here, the imidazolium cation of the protonated His-Asp couple should be highly effective, and an important function of the local environment in the enzyme–substrate complex may be to prevent this cation from becoming deprotonated.

There are three reasonable roles of the aspartyl carboxylate group in this series of steps. One would be to favor the proper imidazole tautomer, another to orient the imidazole ring throughout the reaction to best advantage, and the third to offer some assistance to stabilizing the imidazolium cation. A possible measure of the last of these roles is the moderately downfield ^{15}N shift (191.6 ppm) of N3 (τ) of α-lytic protease in acidic solutions compared to N3 shifts (average of about 200 ppm) of the other imidazoles listed in Table I. This shift difference may indicate some degree of charge transfer to the carboxylate group, but judging from the N3 shift of unprotonated 1-methylimidazole (128.5 ppm), it is unlikely that charge transfer occurs to the extent of much more than 10%. Such an amount of charge transfer is about what might be expected for a carboxylic acid nitrogen-base pair in which the pK_a of the acid is 4 units less than that of the conjugate acid of the base.[45] Consequently, there is no need to postulate reversed pK_a values for the histidyl and aspartyl residues to account for these shifts.

That there may be some degree of stabilization arising from the imidazolium–carboxylate interaction in α-lytic protease (analogous to **6**) seems possible from the fact that the pK_a of the histidyl residue in this enzyme is 7.0, even though many histidyl residues in other proteins have pK_a values less than 6.[23]

The formulation we have given of the progress of peptide hydrolysis as far as serine ester suggests that decomposition of the tetrahedral intermediate to the serine ester plus amine could be the overall rate-determining step for serine protease cleavage of peptide bonds and, if this is so, there is no kinetic requirement for postulating a charge-relay mechanism. The formulation also requires that a tetrahedral intermediate "accumulate" but, because our estimates show that both **9** and **11** are likely to be substantially less stable than the starting amide and alcohol, such accumulation in the enzymatic reaction would be very small, except where the substrate is so constituted as to produce a stabilized tetrahedral intermediate which decomposes more slowly to serine ester plus amine than it is formed.[46]

To summarize, the histidine of the catalytic triad of α-lytic protease appears to have a base strength which is essentially normal for an imidazole derivative but, in the pH range where the enzymatic activity is high, the histidine tautomer is favored with the hydrogen located on N3(π), as the result of hydrogen bonding to the aspartate anion and possibly the serine hydroxyl. Thus, the ^{15}N NMR shifts, like the proton shifts,[22] support the general geometry postulated for the charge-relay mechanism but not the idea of an unusually weakly basic histidine or an unusually strongly basic aspartate carboxylate anion.

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research, but we should emphasize that they are in no way responsible for its deficiencies either in the experiments or our interpretation of the results. We also appreciate the myxobacter 495 culture supplied by Dr. Hunkapiller, from which the auxotroph was derived.

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EPR and NMR Combined Analysis of the Metal-ATP Interaction

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Abstract: The Mn(II)-ATP system has been investigated at the light of new electron spin relaxation findings. A dynamic model, taking into account a distribution of correlation times, is suggested. Namely transverse and longitudinal nuclear relaxation times are analyzed in connection with new values of the electron spin relaxation time. A reinvestigation of NMR and EPR results is tempted as a consequence of the new model. The combined NMR and EPR analysis allows an unambiguous interpretation of nuclear paramagnetic relaxation rates and points out the direct involvement of N(7) in the metal binding.

The metal ion-ATP interaction has been the subject of extensive research,[1-6] due to its relevance in several enzymatic reactions which require nucleoside triphosphate as activator.

Among the various metal ions, Mn(II) has been the most investigated one since Mg(II) ⇄ Mn(II) isomorphous replacement is possible and the Mn(II)-ATP complex has its own biological activity.[7]

Nuclear magnetic resonance,[3-6,8,9] temperature jump,[10] and electron spin resonance[11-13] have been used to elucidate the nature of the interaction both from the structural and the dynamic points of view.

A backbound phosphate-metal ion ring complex has been confirmed by several authors[3,8,9] as the actual substrate for the enzyme. Nevertheless, it is controversial whether the metal ion is water-bridged to N(7) or directly bonded to it.

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