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Carbon Nuclear Magnetic Resonance Studies of the Histidine Residue in α-Lytic Protease. Implications for the Catalytic Mechanism of Serine Proteases†

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ABSTRACT: Selective ^{13}C enrichment of C-2 of the single histidine residue of the serine protease α-lytic protease has allowed direct study of the Asp-His-Ser catalytic triad. Both the chemical shift of C-2 and the coupling between C-2 and its directly bonded hydrogen have been observed as a function of pH. We interpret the results to indicate that only below pH 4 does the imidazole ring of the histidine residue become protonated and only above pH 6.7 does the aspartic acid residue lose a proton to generate a carboxylate anion. Thus, over the pH range 4–6.7, the catalytic triad consists of a *neutral* aspartic acid and a *neutral* histidine residue—not the ionized forms hitherto assumed. These new assignments for the ionization characteristics of the aspartic acid and histidine residues of the catalytic triad lead to a proposed catalytic mechanism that avoids any requirement for unfavorable charge separation. In this view, the histidine residue plays two roles: (i) it provides insulation between water and the buried carboxylate anion of the aspartate, thus ensuring the carboxylate group a hydrophobic environment, and (ii) it provides a relay for net transfer of protons from the serine hydroxyl to the carboxylate anion. The aspartate anion acts as the ultimate base which holds a proton during catalysis. An anionic, rather than a neutral, base has advantages; it both avoids the necessity of charge separation and, by giving the catalytic locus an overall negative charge, assists preferential expulsion of product relative to substrate from the active site of the enzyme. Relaxation measurements (T_1, T_2, and nuclear Overhauser enhancement) indicate that, over the pH range of enzymic activity, the histidine residue is held rigidly within the protein.

From the first discovery (Matthews *et al.*, 1967; Blow *et al.*, 1969) of a buried carboxylate anion as part of a precisely arranged catalytic triad of residues (consisting of the carboxylate anion of aspartate, the imidazole ring of histidine, and the hydroxyl group of serine), the detailed nature of the charge relays and accompanying proton transfers that occur during catalysis by serine proteases has been the focus of considerable interest. This array of three residues occurs in many enzymes which show clear homologies and most likely constitute a case of divergent evolution of a single family of proteins. (Members of this family include, for example, chymotrypsin, trypsin, elastase, and α-lytic protease [Olson *et al.*, 1970].) Even in enzymes which possess no other apparent homology, and are therefore probably unrelated in terms of their ultimate origins (such as the chymotrypsin family on the one hand and the subtilisin family on the other), the striking presence of this identical catalytic triad provides evolutionary testimony of its unique catalytic efficacy. The question of the true microscopic ionization behavior of the participants of this triad has, however, hindered the realization of a completely satisfactory account of the catalytic act.

^{13}C Nmr for Study of Proteins. Nuclear magnetic resonance (nmr) allows study of the environment of individual nuclei of molecules in solution and has recently found increasingly fruitful application to problems of biological importance. Meadows *et al.* (1967, 1969) used proton magnetic resonance to study ionization of the histidine residues in ribonuclease and the effect of inhibitor binding on the two catalytic histidine groups of the enzyme. Wuthrich *et al.* (1968) and Ogawa and Shulman (1971) observed those proton resonances of hemoglobin which are shifted by the ring currents of the porphyrin ring and thereby studied the effects on protein conformation of ligand binding to the heme groups. However, the relatively narrow range of proton chemical shifts and the resultant lack of dispersion usually require exchange with deuterium oxide to remove amide protons from the backbone peptide bonds and thereby expose signals from other protons of interest.

Groups containing fluorine nuclei have been covalently attached to proteins. Subsequent study by ^{19}F spectroscopy has been reported, for example, for hemoglobin and ribonuclease by Raftery *et al.* (1972) and Huestis and Raftery (1972). Though, in the cases mentioned, appropriate control studies showed only insignificant change in protein function as a result of attachment of label, such techniques may potentially cause unknown alterations in protein conformations.

Carbon magnetic resonance offers great potential for studies

† Contribution No. 4699 from the Church Laboratories of Chemical Biology, California Institute of Technology, Pasadena, California 91109. *Received June 13, 1973.* We wish to acknowledge generous financial support of this research by grants from the U. S. Public Health Service, NIGMS-16424 and NIGMS-10218, and the Medical Research Council of Canada, MA-4409.

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of proteins because of the wide chemical shift range of ^{13}C (Horsley *et al.*, 1970). However, the low natural abundance and relatively low sensitivity of ^{13}C usually require unattainably long signal accumulation times in order to observe single resonances from proteins even though recent increases in probe sizes have dramatically improved achievable signal-to-noise levels for dilute solutions of proteins (Allerhand *et al.*, 1972). Nevertheless, natural abundance ^{13}C studies have been productively applied to protein studies (Moon and Richards, 1972a).

Specific ^{13}C enrichment of functionally important nuclei has great promise and is the technique used in the work reported in this paper. In this case, the low natural abundance ^{13}C background becomes an advantage as it usually allows the unambiguous observation of the enriched site(s). Moreover, replacement of ^{12}C by ^{13}C leads to only negligible changes in the structure or action of a protein.

Chaiken *et al.* (1973) synthesized the S-peptide of ribonuclease with uniformly ^{13}C-enriched phenylalanine and studied the complex between this S-peptide and the remaining fragment of the enzyme. (Though not the native enzyme, the complex possesses 15% catalytic activity.) Browne *et al.* (1973) enriched the C-2 carbon of the imidazole ring in each of the four histidine residues of the α subunit of tryptophan synthetase from *E. coli* but were unable to resolve the overlapping signals from the four ^{13}C nuclei in the four histidine residues. Moon and Richards (1972b) used ^{13}CO as a ligand of the heme groups in myoglobin and various hemoglobins to study the environment experienced by the bound ligand.

The study reported in this paper represents the first specific ^{13}C enrichment of a single carbon atom in a native protein together with a study of the magnetic resonance parameters of this carbon. The C-2[1] carbon of the imidazole ring of the single histidine residue of the bacterial serine protease α-lytic protease from Myxobacter 495 (Whitaker, 1970) was enriched in ^{13}C by growing the bacteria in a medium containing L-[2-^{13}C]histidine.

We chose α-lytic protease as the subject of this study for four reasons. (i) α-Lytic protease has a bacterial origin. This facilitates biosynthetic incorporation of labeled amino acids. (ii) α-Lytic protease possesses only a single histidine residue. In contrast, chymotrypsin has two, trypsin has three, and elastase and subtilisin have six histidine residues. Therefore, the behavior of the single histidine of the catalytic triad of α-lytic protease can be observed without possibly confusing complications caused by the presence of other histidine residues. (iii) α-Lytic protease exhibits remarkable stability toward denaturation and autolysis even in concentrated solutions and under conditions of pH and temperature where enzymic activity is maximal. This stability is unparalleled by other well-studied proteolytic enzymes and is essential for ^{13}C magnetic resonance because of the time required for signal accumulation. (iv) The enzyme is a homolog of mammalian serine proteases, a family of proteolytic enzymes each of which possesses a catalytic triad of side-chain groups consisting of the carboxyl group of an aspartic acid (residue 102 in the amino acid sequence of chymotrypsinogen), the imidazole ring of a histidine (residue 57 in chymotrypsinogen), and the hydroxyl group of a serine (residue 195 in chymotrypsinogen). As such, α-lytic protease serves as a paradigm for this great group of enzymes.

The concept of the close relationship of α-lytic protease to the mammalian serine proteases finds support in the extensive sequence homology between α-lytic protease on the one hand and chymotrypsin, trypsin, and elastase on the other (Olson *et al.*, 1970). In fact, McLachlan and Shotton (1971) were able to construct a tentative three-dimensional model of α-lytic protease based on the extensive sequence homology between α-lytic protease and elastase and the known three-dimensional structure of elastase. In so doing, they formed a catalytic core which displayed the same relative orientation of the three components of the catalytic triad that characterizes the mammalian enzymes. The sequence homology α-lytic protease shares with the mammalian proteases distinguishes it from another bacterial enzyme, subtilisin, which, though having a structurally and functionally identical catalytic locus (Robertus *et al.*, 1972), lacks any discernible sequence homology with the mammalian enzymes.

In specificity, α-lytic protease mimics elastase. Both possess elastolytic and bacteriolytic activity, and both preferentially hydrolyze amide bonds whose carbonyl groups belong to alanine or valine (Kaplan *et al.*, 1970). Also both enzymes bind neutral, specific substrates independently of pH over the range pH 5–10 (Kaplan *et al.*, 1970). In contrast, substrate binding to trypsin and α-chymotrypsin decreases drastically at higher pH with an apparent pK_a of 8.5–9.5 (Oppenheimer *et al.*, 1966; Fersht and Requena, 1971). This decrease in binding at high pH in chymotrypsin reflects deprotonation of the N-terminal amino group of Ile 16 which forms a salt bridge with Asp 194; disruption of this Ile-Asp salt bridge leads in turn to destruction of the specificity binding pocket. Elastase and α-lytic protease, on the other hand, show no such dependence of binding on ionization of an amino terminus presumably because, in these enzymes, the region analogous to the specificity pocket in trypsin and chymotrypsin is filled with side-chain residues of the enzymes themselves. As a result of this absence of a specificity pocket in elastase and α-lytic protease, binding is relatively weak over the entire pH range, and this binding is, therefore, not significantly affected by deprotonation of an N-terminal amino group.

α-Lytic protease also reacts with irreversible inhibitors as do other serine proteases. Thus, the active serine residue of α-lytic protease reacts irreversibly with diisopropyl fluorophosphate, a typical inhibitor of serine proteases, to yield an inactive enzyme (Whitaker and Roy, 1967). In contrast to the active site histidine residues of chymotrypsin and trypsin (which react with the chloromethyl ketone derivatives of tosyl-L-phenylalanine and tosyl-L-lysine, respectively), those of elastase and α-lytic protease fail to react with tosyl-L-alanine chloromethyl ketone (Kaplan *et al.*, 1970). However, Thompson and Blout (1973) have recently reported that the active site histidine of elastase does react with *N*-Ac-L-Pro-L-Ala-L-Pro-L-Ala chloromethyl ketone.

α-Lytic protease exhibits kinetic properties remarkably similar to those of mammalian enzymes (Whitaker, 1970; Kaplan and Whitaker, 1969). These enzymes all manifest a similar deuterium isotope effect. In deuterium oxide the value of k_{cat}/K_M is only one-third that observed in aqueous solution for enzyme-catalyzed hydrolysis of appropriate *N*-acetylamino acid esters. (For ester hydrolysis catalyzed by these enzymes, deacylation is rate limiting.) Moreover, in all these cases, k_{cat}/K_M depends upon an ionization of a group in free enzyme with an apparent pK_a of 6.7.

Based on these striking similarities in sequence homology, specificity, reactivity toward irreversible inhibitors, and catalytic behavior, we conclude that α-lytic protease is a representative member of the family of serine proteases. Findings

[1] This carbon can also be designated the $C^{\epsilon 1}$ of histidine.

about its mechanism of action will, therefore, be generally applicable to the other serine proteases.

In addition to α-lytic protease, Myxobacter 495 also produces an enzyme containing zinc, β-lytic protease, an endopeptidase possessing eight histidine residues. This provides another protein which becomes enriched with ^{13}C during growth on a medium containing L-[2-^{13}C]histidine and allows study of the C-2 of histidine residues in a random-coil polypeptide when the protein is denatured.

Experimental Section

Materials. L-[2-^{14}C]Histidine (lot CFA 137-56, 50 Ci/mol) was purchased from Amersham-Searle. Potassium cyanide [91.4% ^{13}C] (lot 8X43) was obtained from Prochem. Amberlite IR-68 (20–50 mesh), IR-120 (20–50 mesh), and CG-50 (200–400 mesh) were purchased from Mallinckrodt, and Bio-Rad AG11A8 (50–100 mesh) was obtained from Bio-Rad. Monosodium glutamate, sucrose, and salt-free casein hydrolysate was purchased from Isolab. *N*-Benzoyl-L-alanine methyl ester was synthesized from benzoyl chloride and L-alanine methyl ester hydrochloride according to the procedure of Whitaker (1970) (mp 58.5–59°, lit. mp 58–59°).

L-[2-^{13}C]Histidine was synthesized from 18.2 g of L-2,5-diamino-4-ketovaleric acid dihydrochloride and 7.4 g of potassium thiocyanate [91.4% ^{13}C] according to the method of Ashley and Harrington (1930) and Heath *et al.* (1951). The product was isolated as the free base by passing the crude reaction product down a column of Bio-Rad AG11A8 ion retardation resin with distilled water as eluent. The water was removed by lyophilization and the histidine was crystallized from ethanol–water (yield 2.4 g, 20% based on $KS^{13}CN$). The proton and cmr spectra were correct for L-histidine enriched with 91% ^{13}C at C-2.

Growth of Myxobacter 495. The procedure described by Whitaker (1970) for culturing Myxobacter 495 was modified to increase enzyme production and decrease dilution of L-[2-^{13}C]histidine by histidine in the casein hydrolysate used as nutrient. The Casamino acid concentration of the culture medium was reduced from 20 to 0.5 g/l., and monosodium glutamate (20 g/l.) served as the new nitrogen source. The inorganic salt content of the medium consisted of $K_2HPO_4 \cdot 3H_2O$ (2 g/l.), NaCl (2 g/l.), $MgSO_4 \cdot 7H_2O$ (1 g/l.), ferric sulfate (25 mg/l.), $ZnSO_4 \cdot 7H_2O$ (4 mg/l.), and $MnSO_4 \cdot H_2O$ (3 mg/l.). Sucrose (10 g/l.) replaced the glucose used with the Casamino acid medium. Solutions of the amino acids and inorganic salts in tap water were mixed in 2800 ml of Fernbach culture flasks fitted with cotton plugs and autoclaved at 121° for 20 min. Sucrose solutions in distilled water were autoclaved separately and added to the culture flasks before inoculation.

Freeze-dried cultures of Myxobacter 495 were stored at −20°. They were transferred to flasks containing 50 ml of culture medium and incubated at 27° for 48 hr on a rotary shaker describing a circle of 1-in. radius at 100 rpm. These 50-ml cultures were used as inocula for the Fernbach flasks containing 1 l. of medium. The Fernbach cultures were then incubated with shaking for 84 hr. For preparations of ^{13}C- and ^{14}C-enriched enzyme, 1 ml of a sterile 5% solution of labeled histidine was added to each Fernbach flask at 0, 20, 40, and 60 hr after inoculation.

Purification of α- and β-Lytic Protease. The procedure for enzyme purification described by Whitaker (1970) was modified to increase the ease and efficiency of isolating enzyme from a small volume of culture medium. All purification procedures were carried out in a cold room at 4°.

Six liters of 84-hr culture was centrifuged (8000 rpm, 30 min) to remove bacteria. Sixty milliliters (settled volume) of Amberlite IR-68 (acetate form) and 50 ml of Amberlite IR-120 (ammonium form) were added to the supernatant, the mixture was stirred for 2 hr, and the solution was filtered through nylon mesh to remove the mixed-bed ion exchange resins. The filtrate was titrated to pH 4.95 by addition of 20% acetic acid, and 100 ml of Amberlite CG-50 (equilibrated with 0.10 M sodium acetate buffer (pH 4.95)) was added to it. The mixture was stirred for 24 hr, and the resin was collected by filtration on a coarse-frit sintered-glass funnel. The resin was washed on the funnel with 2 l. of pH 4.95 acetate buffer and transferred to a beaker containing 100 ml of 0.033 M sodium citrate buffer (pH 6.25). It was then titrated to pH 6.40 by slow addition (with stirring) of 0.5 N sodium hydroxide. The resin was washed on the funnel with 1 l. of pH 6.25 citrate buffer and added to a 2-cm diameter chromatography column previously packed with 100 ml of Amberlite CG-50 (equilibrated with pH 6.25 citrate buffer). The column was washed successively with 250 ml of pH 6.25 buffer, 1 l. of pH 5.88 0.16 M sodium citrate buffer, and 1 l. of pH 6.20 0.27 M sodium citrate buffer. β-Lytic protease and α-lytic protease were eluted by the pH 5.88 buffer and pH 6.20 buffer, respectively. The two enzyme fractions were then titrated to pH 4.95, adsorbed onto 20 ml of Amberlite CG-50, and chromatographed as before. The rechromatographed fractions were exhaustively dialyzed against distilled water and lyophilized. The yields of freeze-dried enzyme were 600 mg of α-lytic protease and 125 mg of β-lytic protease. Each enzyme gave a single, sharp band on polyacrylamide disc gel electrophoresis at pH 8.3 (Ornstein, 1964). The α-lytic protease was assayed for esterase activity against *N*-benzoyl-L-alanine methyl ester (0.01 M in 0.1 M KCl at 25°) and showed a value of $k_{cat}/K_M = 740\ M^{-1}\ sec^{-1}$ (based on enzyme $E_{278}^{1\%} = 8.9$) compared to the previously reported value of 723 $M^{-1}\ sec^{-1}$ (Whitaker, 1970).

L-[2-^{14}C]Histidine Incorporation into α-Lytic Protease. Solutions of enzyme (~10 mg/ml) produced in a medium supplemented by 200 mg of L-[2-^{14}C]histidine (24.6 μCi/mmol; 5.42×10^7 dpm/mmol) were added to Scintisol Complete scintillation fluid and counted in a Packard TriCarb liquid scintillation spectrometer, Model 3375. The counts were corrected for background and counting efficiency and were used to calculate the specific radioactivity of the enzyme.

Samples of enzyme (~5 mg) were dissolved in 1 ml of constant-boiling hydrochloric acid, degassed, sealed under vacuum, and heated at 110° for 24 hr. The hydrolyzed samples were diluted with water and dried *in vacuo* over potassium hydroxide pellets to remove excess acid. The hydrolysate was then subjected to high voltage paper electrophoresis at pH 6.5. The amino acids were separated into five bands: aspartic acid, glutamic acid, neutral amino acids, histidine, and lysine + arginine. The bands were eluted with 1% acetic acid, and the amino acid concentration and specific radioactivity of each were determined. Only the histidine band showed significant radioactivity (2.2×10^7 dpm/mmol); all other bands had less than 250 dpm/mmol.

Nmr spectra were recorded on a Varian XL-100-15 nmr spectrometer operating at 25.17 MHz in the Fourier transform mode. Data accumulation and Fourier transformation of the free induction decay were carried out by a Varian 620 i computer interfaced to the spectrometer. In general, spectra were taken at a 2000-Hz sweep width using a 90° pulse (150 μsec) and an acquisition time of 0.15 sec. A sensitivity enhancement of 0.04–0.10 sec was applied to the free induction decay before the Fourier transformation was carried out.

Proton noise decoupling was used in all cases except determination of coupling constants and nuclear Overhauser enhancements.

Study of Titration of Imidazole Groups by Carbon Magnetic Resonance. α-Lytic protease solutions (5–6 mM) were made up in 0.2 M KCl and transferred to a 12 mm o.d. nmr tube fitted with vortex plugs and a D_2O locking capillary. The pH was measured with a Radiometer Model PM 26 pH meter equipped with a Radiometer Model GK2322C combination electrode that could be inserted into the nmr tube. The pH was checked before and after each spectrum was recorded, and the two values always agreed to within ±0.05 pH. The pH was varied by addition of 1 N KOH or 1 N HCl. Activity of the enzyme against *N*-benzoyl-L-alanine methyl ester was unchanged (±5%) during recording of the spectra.

The low water solubility of native β-lytic protease precluded nmr studies of the active enzyme. However, the protein was denatured and solubilized by suspending it in 1 N KOH for 24 hr at room temperature. The solution was titrated to pH 8 with 1 N HCl, dialyzed exhaustively against distilled water, and freeze-dried. The protein (95 mg) was then dissolved in 1.5 ml of 0.2 M KCl, and the cmr spectrum of this solution was recorded at several values of pH over the range pH 4.6–8.2 (pH adjusted with 1 N KOH or 1 N HCl).

T_1 Measurements. The spin–lattice relaxation time, T_1, was determined by the progressive saturation method (Freeman and Hill, 1971). T_1 values were calculated by an iterative computer fit of the relative peak intensities, S_a/S_b, and the pulse intervals, a and b, according to the formula

$$S_a/S_b = (1 - e^{-a/T_1})/(1 - e^{-b/T_1})$$

Nuclear Overhauser Effect Measurements. NOE values (Kuhlmann *et al.*, 1970) were measured from the relative areas of the proton coupled and decoupled spectra. A 90° pulse and a pulse interval of $\sim 4T_1$ were used. Spectra were plotted using the absolute intensity scale of the Varian 16K Fourier transform program. Peak areas were determined by computer integration or by Xeroxing the spectra, cutting out the peaks, and weighing them.

Results

Incorporation of Histidine into α-Lytic Protease. As a preliminary test for (i) dilution of added histidine by endogenous sources and (ii) significant operation of metabolic pathways which transfer label originally present in C-2 of added histidine into other amino acids, we studied the incorporation of L-[2-^{14}C]histidine into α-lytic protease. Addition of labeled histidine to the culture medium of wild Myxobacter 495 in several aliquots at intervals during the growth cycle gave reasonable incorporation of label without significant transfer to other amino acid residues. Specifically, α-lytic protease isolated from a medium supplemented by 200 mg of L-[2-^{14}C]histidine (5.42×10^7 dpm/mmol; added in four equal aliquots 0, 20, 40, and 60 hr after inoculation) had a specific activity of 2.08×10^7 dpm/mmol (dilution to 40% of the original activity). The resulting protein was hydrolyzed with acid and the hydrolysate was separated into different amino acid fractions by paper electrophoresis. Appreciable radioactivity appeared only in the histidine band (2.2×10^7 dpm/mmol); the other bands all showed less than 250 dpm/mmol. We accordingly conclude that no label originally present at C-2 of the added histidine is transferred to any other amino acid residue (at least not without very extensive dilution) by the metabolic activities of the microorganism.

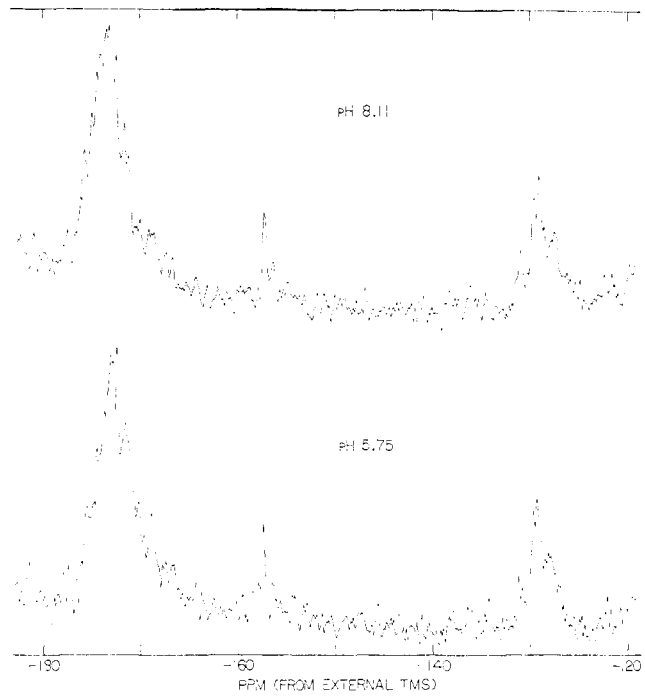


FIGURE 1: Proton noise decoupled cmr spectra of α-lytic protease: 6 mM enzyme, 34°, 0.2 M KCl. Each spectrum represents 250,000 transients at 0.20 sec acquisition time, 2000 Hz sweep width, and 90° pulse.

In the experiments involving addition of ^{13}C-labeled histidine, label in the isolated α-lytic protease had been diluted only to about 65% of its original concentration (91.4% enriched L-[2-^{13}C]histidine was added and the isolated α-lytic protease is judged to have a ^{13}C enrichment in C-2 of the histidine residue of about 60%).

Magnetic Resonance Parameters. Figure 1 shows two proton noise-decoupled natural abundance cmr spectra in the low-field region of α-lytic protease at pH 5.8 and 8.1. The resonances may be assigned from the data of Horsley *et al.* (1970). The signals from the ring carbons of the six phenylalanine and four tyrosine residues of α-lytic protease are evident 129 ppm downfield from external tetramethylsilane. The guanidinium carbons of the 12 arginine residues produce the sharp signal at −157.25 ppm. The broad signal centered at −173 ppm represents the carbonyl carbons of the peptide backbone.

Figure 2 shows three comparable cmr spectra (at pH 5.6, 6.5, and 8.5) for α-lytic protease containing histidine enriched with ^{13}C at C-2 (approximately 60% enrichment). A new peak, which we assign to the C-2 carbon of the single histidine residue, appears at −136 ppm ($\nu_{1/2} \sim 30$ Hz). In other aspects, the spectra of natural abundance and L-[2-^{13}C]histidine α-lytic protease are identical.

Incorporation of L-[2-^{13}C]histidine into α-lytic protease by Myxobacter 495 also yields enriched β-lytic protease which, when denatured, provides a useful comparison for the magnetic resonance parameters of histidine residues in a random-coil polypeptide. The cmr spectrum of denatured β-lytic protease shows a strong resonance ($\nu_{1/2} \sim 10$ Hz) around −135 ppm which we assign to the eight C-2 carbons of the eight histidine residues of the protein.

The upfield resonance of the doublet from the histidine C-2 carbon in proton coupled cmr spectra of α-lytic protease is

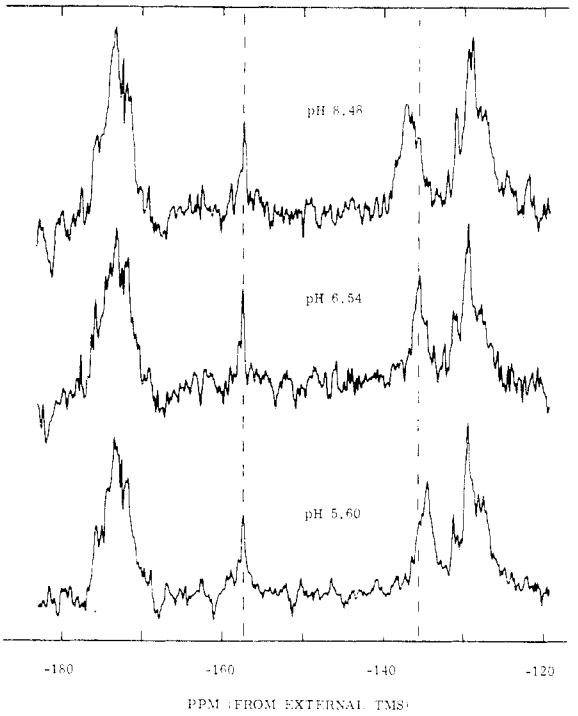


FIGURE 2: The ^{13}C enriched, proton noise decoupled cmr spectra of α-lytic protease: 6 mM enzyme, 34°, 0.2 M KCl. Each spectrum represents 200,000 transients at 0.20 sec acquisition time, 2000 Hz sweep width, and 90° pulse.

partially obscured by the signals from the other aromatic carbons (Figure 3). This interference can be eliminated by computer subtraction of a proton coupled natural abundance ^{13}C spectrum taken under identical conditions from the ^{13}C-enriched spectrum. This process yields a simplified spectrum consisting only of the doublet from the histidine C-2 from which $^1J_{CH}$ can be measured directly. Alternatively, $^1J_{CH}$ values for the C-2 carbon can be calculated from the difference between the position of the resonance in the decoupled

TABLE I: Chemical Shifts and Directly Bonded Carbon–Hydrogen Coupling Constants for C-2 Carbon in Imidazole Derivatives.[a]

Compound	δ (ppm (±0.04) from Me_4Si)		$^1J_{CH}$ (Hz (±1))	
	Cation	Neutral	Cation	Neutral
Imidazole	−134.05	−136.23	219	209
4-Methylimidazole	−133.17	−135.40	221	208
4-Methylimidazole (dioxane)	−133.13	−134.49	219	205
1-Methylimidazole	−135.52	−138.38	220	207
L-Histidine methyl ester	−135.08	−136.71	222	208
N-Acetyl-L-histidine	−134.17		221	
	−133.85	−139.45	220	204
(4-Imidazolyl)-acetic acid	−134.25		220	
	−133.65	−136.47	221	205
β-Lytic protease[b] (denatured)	−134.09	−136.67	218	206

[a] Measured for 1–2 M aqueous solutions unless otherwise indicated. [b] 1–2 mM solution in 0.2 M KCl.

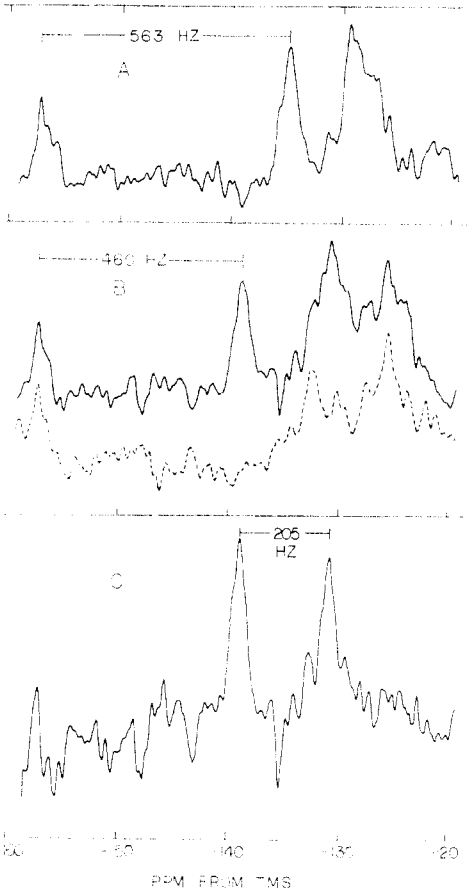


FIGURE 3: Measurement of $^1J_{CH}$ for histidine C-2 in α-lytic protease at pH 5.98: (a) proton decoupled cmr spectrum, ^{13}C enriched, 50,000 transients; (b) (——) proton coupled cmr spectrum, same sample as in (a), 250,000 transients; (- - - - -) proton coupled cmr spectrum, natural abundance ^{13}C, 250,000 transients; (c) difference spectrum obtained by computer subtraction of the natural abundance spectrum (- - - - -) from ^{13}C enriched spectrum (——) of (b). $^1J_{CH}$ can be calculated either directly from the difference spectrum (c), $^1J_{CH}$ = 205 Hz, or by multiplying by a factor of 2 the difference between the chemical shifts of the decoupled resonance (a) and the downfield resonance of the coupled spectrum (b). $^1J_{CH}$ = 2(563 − 460) = 206 Hz.

spectrum and the position of the downfield peak of the doublet in the coupled spectrum taken of the same sample. Both positions can be measured accurately relative to the sharp arginine signal whose position never varied more than 1 Hz from external D_2O lock in any of the spectra we have recorded. Although this alternate method of measurement yields a somewhat larger error (since the difference in peak positions must be multiplied by two to yield $^1J_{CH}$), six determinations in the pH range 5–6 have yielded a reproducible value for $^1J_{CH}$ (203, 204, 205, 205, 206, and 208 Hz). Figure 3 shows the spectra used for calculation by both of the above methods for $^1J_{CH}$ at pH 5.98.

Table I collects the carbon chemical shifts (δ) and coupling constants ($^1J_{CH}$) for the C-2 carbons of imidazole and some of its derivatives. Table II lists the δ and $^1J_{CH}$ values for the C-2 carbon of the histidine residue in α-lytic protease at several values of pH. Table III lists the relaxation parameters for the C-2 carbon of the histidine residue of α-lytic protease at pH 5.8 and 8.2.

Titration Results. The chemical shifts and $^1J_{CH}$ constants of the ring carbons depend on the state of protonation of the nitrogen heteroatoms of the ring and accordingly vary with pH. The chemical shift may also be significantly affected by

TABLE II: Chemical Shift and Coupling Constant Values for C-2 Carbon in Histidine Residue of α-Lytic Protease.

pH	Chemical Shift (ppm ± 0.12 from Me_4Si)	$^1J_{CH}$ (Hz ± 3)
8.2	−136.95	205
5.2	−134.57	205[a]
3.3	−134.81	208
	−134.05	222
	−132.46	218

[a] Six determinations of $^1J_{CH}$ around pH 5–6 yielded values of 203, 204, 205, 205, 206, and 208 Hz.

the external environment experienced by the carbon nuclei. If the rate of proton acquisition or proton loss by the nitrogen atom or changes in the environment around the ring occur rapidly on the nmr time scale, the observed spectrum will represent the average of spectra for the relative populations of each species (Pople *et al.*, 1959). The condition for this "fast exchange" is that $\tau_A < \sqrt{2}/2\pi\Delta_{AB}$ where τ_A is the mean lifetime of the shorter lived species. For $\Delta_{AB} \sim 60$ Hz, this requires $\tau_A < 3.5 \times 10^{-3}$ sec, a condition easily satisfied for simple ionization of imidazole and its derivatives in aqueous solution and one that also characterizes the enzyme spectra reported here except for those of α-lytic protease below pH 4. Thus, we observe a single resonance of essentially constant line width that shifts as a function of pH for denatured β-lytic protease and for α-lytic protease between pH 5 and 9.

Under conditions of fast exchange, the observed chemical shift, δ, will vary with pH in the following manner

$$(\delta_{obsd} - \delta_a)/(\delta_b - \delta_a) = K_a/([H^+] + K_a)$$

where δ_a and δ_b are the chemical shifts of the two exchanging species, respectively, and K_a is the acid ionization constant of some ionization (either that of the imidazole itself or a neighboring group) which perturbs the environment experienced by the imidazole. Figure 4 collects the observations of the chemical shifts as a function of pH of the C-2 carbon of the single histidine residue of native α-lytic protease between pH 4 and 10. The solid line in this figure represents a theoretical curve calculated by an iterative computer fit of the experimental points to the above equation. The excellent fit between the experimental points and the theoretical curve based on a simple one group ionization ($pK_a = 6.7$) suggests that such an ionization does occur in this pH range. However, we observe that $^1J_{CH}$ for C-2 of the histidine residue has a value characteristic of a *neutral* imidazole ring from pH 5.2 to 8.2

TABLE III: Nmr Relaxation Parameters for His 57 C-2 Carbon in α-Lytic Protease.[a]

pH	$\nu_{1/2}$ (Hz)	T_1 (sec)	NOE	τ_c (sec)
5.8	28 ± 3	0.063 ± 0.002	1.16 ± 0.10	1.8 ± 0.1 × 10^{-8}
8.2	32 ± 3	0.060 ± 0.015	1.18 ± 0.10	1.7 ± 0.1 × 10^{-8}

[a] Enzyme concentration, 5 mM; temperature, 34°; 0.2 M KCl. τ_c calculated according to Doddrell *et al.* (1972). T_1 values represent average of three determinations.

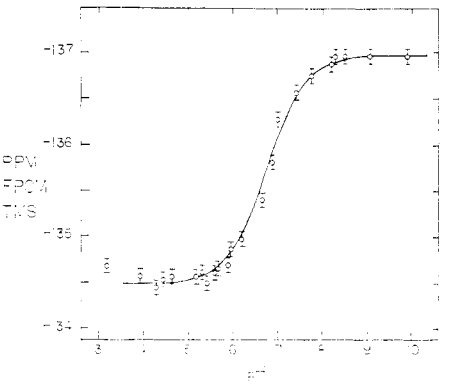


FIGURE 4: Chemical shift of histidine C-2 resonance of α-lytic protease as a function of pH: (○) the chemical shift of C-2 in the neutral histidine residue (see text); (—) theoretical titration curve calculated using pK_a of 6.75 and Δ of 62 Hz.

($^1J_{CH}$ = 205 Hz). Therefore, the ionization ($pK_a = 6.7$) affecting the C-2 chemical shift does not represent ionization of the histidine itself but rather, most likely, that of the neighboring aspartic acid (Asp 102).

Indeed, our interpretation of the spectra of α-lytic protease at pH <4 suggests that only in this pH range does the histidine become protonated. At pH 3.25, three distinct signals are evident which we assign to the histidine C-2 carbon, and they indicate that at this pH the histidine exists in three, slowly exchanging, states (Figure 5). One, which disappears below pH 3, has the same chemical shift (−134.8 ppm), $^1J_{CH}$ (208 Hz) and line width (25 Hz), as the single resonance observed at pH 5.2. This resonance probably represents a neutral histidine within the catalytic triad. A second signal has virtually the same chemical shift (−134.1 ppm) and $^1J_{CH}$ (222 Hz) values as the C-2 carbons of the histidine residues in denatured β-lytic protease. The line width (≤12 Hz) of this signal is considerably narrower than that of the other two (indicating

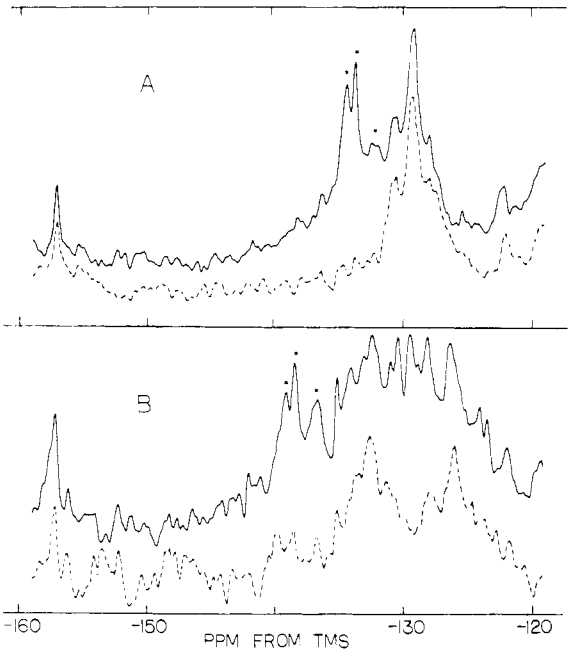


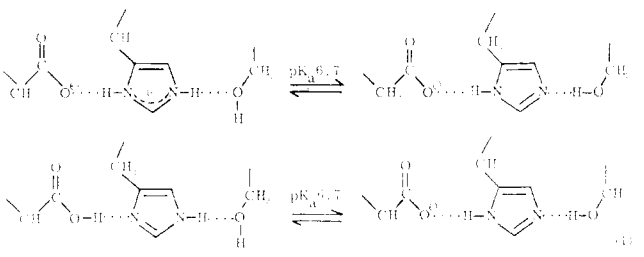
FIGURE 5: Cmr spectra of α-lytic protease at pH 3.25: (—) ^{13}C enriched; (- - - - -) natural abundance ^{13}C; (a) proton noise decoupled; (b) proton coupled. The resonances assigned to C-2 of the histidine residue are marked by an asterisk. Each spectrum represents 250,000 transients at 0.15 sec acquisition time.

significantly more side-chain mobility), and we assign this resonance to a protonated histidine that has been ejected into solution from its normal, partially buried position as a member of the catalytic triad. The third resonance has a chemical shift of −132.46 ppm (2.35 ppm upfield of the first mentioned signal) and a $^1J_{CH}$ (218 Hz) typical of a protonated histidine. This, along with its line width (30 Hz), suggests that it represents the protonated histidine near its normal location in the catalytic triad. In summary, only below pH 4 does the histidine exist in a protonated state, and the two cationic species (one in and one out of the catalytic triad) are in slow exchange with the single neutral form (in the catalytic triad).

Discussion

Selective ^{13}C enrichment of the histidine residue at the active site of α-lytic protease, a homolog of the mammalian serine proteases, has allowed study of the ionization behavior of this residue as a function of pH. Changes in such nmr parameters as chemical shift, coupling constant, relaxation time, and nuclear Overhauser enhancement shed light on the ionizations of the histidine and aspartic acid residues and on the degree of mobility of the imidazole ring within the native protein at various pH's. These results lead to a modification of the generally held views of the role of the catalytic triad and charge transfer in hydrolytic catalysis by serine proteases.

State of Ionization of Histidine and Aspartic Acid Residues as a Function of pH in Serine Proteases. Knowledge of the microscopic ionization behavior of the residues of the catalytic triad is essential to a molecular understanding of catalysis by serine proteases. A great many studies (for example, Fersht and Requena, 1971; Rajender *et al.*, 1971) have shown that an ionization with an apparent pK_a of about 6.7 controls catalytic activity. Unfortunately, none of these studies uniquely defines the state of ionization of the residues of the triad as a function of pH. Thus, either of these situations could describe the available evidence.



This ionization (pK_a = 6.7) is commonly assigned to proton loss from the protonated imidazolium ring (eq 1) of the histidine residue in the catalytic triad (His 57 in chymotrypsin), an assignment which has seemed plausible as the pK_a of histidine side chains in many polypeptides is around 6.4 (Buckingham *et al.*, 1972) and, in general, the pK_a of aspartic acid residues is around 4.5. However, in the conformation of the serine proteases present in crystals, the aspartic acid residue is buried within a hydrophobic region and is not exposed to solvent water (Birktoft and Blow, 1972). Further, in the crystalline enzyme, the histidine is only partially exposed to solvent as N-3 is pointed toward aspartic acid and other residues within the hydrophobic interior of the protein. The environment experienced by the aspartic acid residue and, to a lesser extent, that of the histidine residue also, will be much less polar than water, a situation which would tend to raise the pK_a of aspartic acid above 4.5 and lower the pK_a of histidine below 6.4. Therefore, one should seriously entertain the notion that the microscopic pK_a's of the aspartic acid and histidine residues are significantly perturbed from those expected in aqueous solution. As a result, their pK_a's may well be reversed and the ionization that occurs with $pK_a \sim 6.7$ may *actually reflect net loss of a proton from a neutral aspartic acid carboxyl group in the presence of a neutral imidazole ring* rather than the more commonly accepted loss of a proton from an imidazolium cation in the presence of an aspartate carboxylate anion. Indeed, our interpretation of the nmr data supports the notion that the ionization at pK_a = 6.7 reflects net loss of a proton from aspartic acid.

Nmr Parameters. Two cmr parameters, chemical shift and coupling constant, of the C-2 carbon of an imidazole ring generally respond to the state of ionization of the ring. The data in Table I show that deprotonation of an imidazolium ring leads to downfield shifts of 1.4–5.6 ppm and changes in $^1J_{CH}$ from 220 Hz (cationic) to 207 Hz (neutral). These values agree with those reported for imidazole and its derivatives by Weigert and Roberts (1968), Pugmire and Grant (1968), and Reynolds *et al.* (1973). The C-2 carbons of the eight histidine residues of denatured β-lytic protease on deprotonation exhibit normal changes in chemical shift (−134.1 to −136.7 ppm, a change of −2.6 ppm) and coupling constant ($^1J_{CH}$ = 218–206 Hz) with an apparent pK_a of 6.4. The behaviors of imidazole rings of histidine residues in a denatured protein, therefore, show no significant divergence from the behavior of imidazole rings in small molecules.

The situation for C-2 of the histidine residue of α-lytic protease is, in contrast, complex. The observed changes in chemical shift in the pH range 5–9 can be defined by a single ionization with pK_a = 6.7. However, the fact that $^1J_{CH}$ for the histidine C-2 has a value characteristic of *neutral* imidazole over this entire pH range suggests that the histidine itself does not titrate in this range. Rather, as revealed by the spectra taken at low pH, the histidine ionizes below pH 4. At pH 3.3, for example, three slowly exchanging species are evident from the cmr spectrum of the enzyme. Two of these signals, separated by 2.4 ppm, represent the neutral ($^1J_{CH}$ = 208 Hz) and protonated ($^1J_{CH}$ = 218 Hz) histidine at or near its normal position as a member of the catalytic triad.

Scheme I summarizes the ionization behavior of the aspartic acid and histidine residues of the catalytic triad. Although addition of the first proton (pK_a = 6.7) involves protonation of the aspartic acid, this group does not have to encounter solvent to acquire a proton as one is readily available from the neighboring N-3 nitrogen of the histidine. The histidine can maintain its neutrality by simultaneously acquiring a proton from solvent at its exposed N-1 nitrogen. This process will be expected to be fast on the nmr time scale, and the changes in the C-2 resonance between pH 5 and 9 are consistent with such a rapid exchange process. Addition of a second proton to the catalytic triad must involve reorganization of the three residues owing to the inaccessibility of the aspartic acid and the N-3 of the histidine to solvent. The histidine must first rotate out into solvent to expose N-3 before it can acquire a proton, and this might reasonably be expected to be a relatively slow process (Birktoft and Blow, 1972). The multiplicity of peaks observed in the low pH spectra accords with this description.

In summary, only below pH 4 does the histidine exist in a protonated state. Around pH 5–6, the imidazole ring of the histidine is neutral which implies that the aspartic acid carboxyl group is likewise neutral. The proton added to the catalytic triad with pK_a = 6.7 does not produce an ion-pair

system of carboxylate anion, imidazolium cation (eq 1), but rather a neutral system of carboxylic acid, imidazole (eq 2).

This conclusion should, in fact, occasion little surprise; analogous results can be obtained in model systems when they are in sufficiently nonaqueous environments. For example, equimolar solutions (1–2 M) of acetic acid and imidazole in pure dioxane show no protonation of the imidazole by the acetic acid. Such protonation (forming acetate anion and imidazolium cation) only becomes appreciable on addition of as little as 1–2 molar equivalents of water to the system.

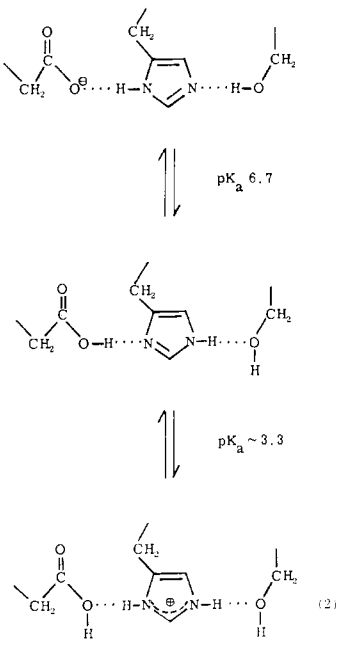
Further, recent X-ray crystallographic results on trypsin (Krieger *et al.*, 1973) show that the interaction between the aspartate anion and neutral imidazole (in crystals of DIP-trypsin grown at pH 7.5) is unusual in that N-3 of the imidazole ring points toward the center of the carboxylate anion. Unlike coordination in solution, this type of interaction seems more typical of that which one might expect in the gas phase or nonaqueous, nonpolar solvents. This observation further supports the unusual character of the aspartate group in the catalytic triad.

Correlation with Other Data. Most techniques used to study ionization within the catalytic triad of serine proteases are inherently incapable of distinguishing between proton loss by an imidazolium cation as distinct from proton loss by a carboxylic acid group. Most kinetic data and other observations of macroscopic ionization behavior can only show that *some* group (or strongly interacting combination of groups) with a $pK_a \sim 6.7$ must be deprotonated to generate catalytically active enzyme.

For example, Fersht and Sperling (1973) reported studies of the net release of protons upon denaturation of α-chymotrypsin in which most of the carboxyl groups (glutamic acid, aspartic acid, and terminal α-carboxyl residues) had been previously blocked. The authors concluded that denaturation of the enzyme at certain pH's led to uptake of one proton by the residues of the catalytic triad. They argued that Asp 102, present as the carboxylate anion in the undenatured, modified protein, was the residue responsible for uptake of this proton. The pK_a of Asp 102 was said to be depressed in the intact enzyme relative to its pK_a in denatured protein. Apart from the manifold uncertainties in accurately measuring quantitative proton uptake in such experiments, they cannot, in principle, define the microscopic ionization of groups involved in strongly interacting systems. (In the present case, for example, a system of Asp-His in which both groups are ionized will give the same result as a system of Asp-His in which both residues are neutral.) Based on their assumption that Asp 102 is anionic and His 57 cationic in the pH 5–6.5 range, Fersht and Sperling argued that the resultant favorable electrostatic interaction must be responsible for a lowered pK_a of Asp 102 and a raised pK_a of His 57. The argument is, however, circular for it assumes that the pK_a of Asp 102 is lower than the pK_a of His 57 (so that the groups are in fact both ionized) to support, in turn, the abnormal pK_a's of these residues. If, in contrast, the pK_a of Asp 102 is *not* intrinsically lower than that of His 57, there will never be such an electrostatic interaction and the pK_a's will be altered only as a result of the environment of the groups.

In fact, the hydrophobic nature of the environment is such that its more likely effect is to raise the pK_a of Asp 102 and lower that of His 57 so that pK_a Asp 102 < pK_a His 57. In this connection, in cysteine proteases such as papain, where the aspartic acid residue is replaced by a neutral asparagine, the active site histidine shows a pK_a around 4.5 (Glazer and Smith, 1971). Further, at least six carboxylic acid side chains

SCHEME I



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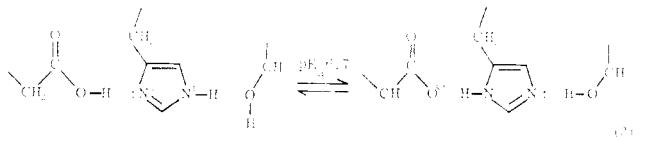
in the proteolytic enzyme pepsin have pK_a's higher than 6 (Edelhoch, 1958). A particularly thorough kinetic study of α-chymotrypsin (Rajender *et al.*, 1971; Lumry and Rajender, 1971) included both observations of the temperature and pH dependence of α-chymotryptic hydrolysis of *N*-acetyl-L-tryptophan ethyl ester. The results showed that the ΔH, ΔS, and "compensation" behaviors of the residue with a pK_a = 6.7 were not those characteristic of ionization of an imidazole but did resemble those for ionization of a carboxylic acid. They suggested that the unusual environment of the histidine accounted for their observations. We suggest that these values are normal and do indeed reflect ionization of the carboxylic acid group of Asp 102.

The very similar inhibition constants for binding neutral and anionic inhibitors in the pH 5–6.5 range also support the suggestion that the active site region is essentially electrostatically neutral in this pH range. For example, K_I values for neutral (*N*-trifluoroacetyl-D-tryptophanamide) and anionic (*N*-trifluoroacetyl-D-tryptophanate) inhibitors are virtually identical between pH 5 and 6.5 (Gammon *et al.*, 1972; Smallcombe *et al.*, 1972; Gammon, 1973). Above pH 7, however, binding of the negatively charged inhibitor decreases drastically while that of the neutral inhibitor is virtually unchanged. This decreased binding of anionic inhibitors at high pH follows easily from the anticipated electrostatic repulsion between a negatively charged active site (because of the carboxylate anion of Asp 102) and a negatively charged inhibitor. However, below pH 6.5 one would have anticipated some favorable electrostatic interaction between the negatively charged inhibitor and the active site if His 57 were in fact protonated and, therefore, positively charged. Though the carboxylate anion of Asp 102 would formally neutralize this positive charge, the imidazolium ring would be sufficiently nearer the inhibitor that some electrostatic effects should be manifested. The absence of such electrostatic effects on binding, therefore, supports the suggestion that in the range pH 5–6.5 both Asp 102 and His 57 are electrically neutral.

Aune and Timasheff (1971), in fact, concluded that the dimerization behavior of α-chymotrypsin reflected a pK_a for His 57 of around 5. They based their assignment on the pH behavior of dimerization in solution and the intermolecular

distances between charged groups observed in the crystalline dimer. They concluded that only the electrostatic interaction between the His 57 of one molecule (which becomes positively charged with a $pK_a \sim 5$) and the α-carboxylate anion of Tyr 146 of the other molecule could reasonably account for the pH dependence of dimerization in solution if the dimer structures in solution and in crystals are identical. Numerous other studies, including those of Faller and LaFond (1971) and Gammon *et al.* (1972), support this proposal.

Cruikshank and Kaplan (1973) used a competitive labeling technique to assign a pK_a of 6.8 to both His 40 and His 57 in α-chymotrypsin which might seem to refute our proposal. However, only that tautomer of His 57 can react which has no proton on the nitrogen exposed to water and, thereby, also exposed to reaction with the labeling reagent (1-fluoro-2,4-dinitrobenzene). Therefore, that tautomer which has a proton on N-1 might well be unreactive toward reagent because, though the imidazole ring is neutral, a reactive nitrogen lone pair is not easily available. (The reactive lone pair is localized on N-3.) Only after ionization of Asp 102, does N-1 of His 57 become reactive (eq 3).



Magnetic resonance observations alledgedly of the proton which is attached to N-3 of the imidazolium ring of His 57, which is hydrogen bonded to the carboxylate anion of Asp 102 and which exchanges only very slowly with solvent, have been reported (Robillard and Shulman, 1972). They observed a broad (~200 Hz) resonance at very low field (−18 to −15 ppm from Me_4Si) in the ^1H spectrum of chymotrypsinogen and δ-chymotrypsin whose chemical shift changed with an apparent pK_a of 7.1. The case of δ-chymotrypsin, especially, presents problems as, under the reported experimental conditions (2–4 mM enzyme, 14°), experience indicates that extensive autolysis, first to α- or γ-chymotrypsin and, further, to catalytically inactive products would occur during the approximately 6 hr used to record each spectrum. We have attempted to duplicate these experiments with chymotrypsinogen, δ-chymotrypsin, and α-lytic protease (which is stable to autolysis) on three different Varian spectrometers (XL-100-15, HR-220, and HR-300) but have been unable to observe the low-field resonance. In any case, their result does not unambiguously distinguish between a proton attached to a carboxyl or to an imidazole group.

They also reported that δ-chymotrypsin inhibited by alkylation of His 57 with tosyl-L-phenylalanine chloromethyl ketone exhibits a low field proton resonance whose chemical shift is unchanged in the range pH 6–8.5. They argued that covalent attachment of the inhibitor raised the pK_a of the modified His 57 above 8.5 because deprotonation of the histidine would force the histidine to swing out toward solution at the same time pulling the phenyl ring of the attached inhibitor out of its hydrophobic binding pocket. The resultant energy cost would prevent deprotonation of the modified His 57 and account for its markedly raised pK_a. However, in our view, if both groups (Asp 102 and modified His 57) were charged, the covalent attachment of the inhibitor to His 57 would, by completely excluding water from the active site, so lower the dielectric constant of this region of the protein molecule that the energetic disadvantage of two charged groups would more than offset the energetic advantage of filling the hydrophobic pocket. However, if above pH 5 Asp 102 and modified His 57 are actually neutral, the reduction in dielectric constant in the active site region could be expected to raise further the pK_a of Asp 102 so that it might not ionize appreciably even at pH 8.5, as observed.

Mobility of Histidine Side Chain. Dipolar interactions between a ^{13}C nucleus and directly bonded ^1H nuclei generally dominate relaxation of the carbon. For small molecules rapidly tumbling in solution, or for freely spinning methyl groups, spin rotation may contribute significantly to the relaxation process. When carbon is directly bonded to a nucleus with a spin greater than $\frac{1}{2}$ (such as nitrogen), scalar relaxation can also be important if the molecule has either a short or an extremely long correlation time. However, both spin rotation and scalar relaxation contribute only negligibly for ^{13}C nuclei having correlation times near the Larmor frequency; in these cases dipolar interactions dominate (Levy and Nelson, 1972).

Doddrell *et al.* (1972) have given a comprehensive theoretical treatment of T_1, T_2, and NOE values for ^{13}C nuclei in molecules with long correlation times. In particular, they have dissected effects on the relaxation times and NOE of a ^{13}C nucleus directly bonded to a single ^1H into those that depend on overall molecular tumbling (characterized by a correlation time τ_R) and those that result from intramolecular reorientations (characterized by a correlation time τ_C).

In the absence of intramolecular reorientation, the molecular tumbling defines T_1, T_2, and NOE as

$$1/T_1 = \frac{1}{10}(\hbar\gamma_H\gamma_C)^2 r^{-6}[\chi_R]$$

$$1/T_2 = \frac{1}{20}(\hbar\gamma_H\gamma_C)^2 r^{-6}[\chi_R + 4\tau_R + (6\tau_R/1 + \omega^2{}_H\tau_R{}^2)]$$

$$\mathrm{NOE} = 1 + 4\phi_R/\chi_R$$

where γ_H is the magnetogyric ratio for hydrogen, ω_H is the Larmor frequency for hydrogen, r is the C–H bond distance (1.07 Å for the C-2 of imidazole)

$$\chi_R = \frac{\tau_R}{1 + (\omega_H - \omega_C)^2\tau_R{}^2} + \frac{3\tau_R}{1 + \omega_C{}^2\tau_R{}^2} + \frac{6\tau_R}{1 + (\omega_H + \omega_C)^2\tau_R{}^2}$$

and

$$\phi_R = \frac{6\tau_R}{1 + (\omega_H + \omega_C)^2\tau_R{}^2} - \frac{\tau_R}{1 + (\omega_H - \omega_C)^2\tau_R{}^2}$$

The effects of internal motion are expressed by the following equations.

$$T_{1R}/T_1 = A + B(\chi_B/\chi_R) + C(\chi_C/\chi_R)$$

$$T_{2R}/T_2 = A + B(T_{2R}/T_{2B}) + C(T_{2R}/T_{2C})$$

$$\mathrm{NOE} = \frac{1 + 4(A\phi_R + B\phi_R + C\phi_R)}{(A\chi_R + B\chi_B + C\chi_C)}$$

where

$$\tau_B^{-1} = \tau_R^{-1} + (6\tau_G)^{-1}$$

$$\tau_C^{-1} = \tau_R^{-1} + 2(3\tau_G)^{-1}$$

$$A = \frac{1}{4}(3\cos^2\theta - 1)^2$$

$$B = 3\sin^2\theta\cos^2\theta$$

$$C = \frac{3}{4}\sin^4\theta$$

In the above equation, θ is the angle between the C–H bond in question and the axis of internal motion. Crystallographic models of serine proteases suggest that the principle motion available to the histidine is to swing in and out from its partially buried position in the catalytic triad. This allowed motion is largely constrained such that it occurs within the plane containing the imidazole ring. The axis of the motion is therefore nearly perpendicular to the plane of the imidazole ring (though not passing through its center) and θ has a value close to 90°.

Assumption of a value of 90° for θ allows calculation for a given value of τ_R of the dependence of T_1, T_2, and NOE on variations in τ_G. Globular proteins with a molecular weight of 20,000 have rotational correlation times, τ_R, between 1 and 2 $\times 10^{-8}$ sec in aqueous solution (Emsley *et al.*, 1965). If there is no independent, internal motion of the imidazole ring in α-lytic protease, the experimental results at pH 5.8 and 8.2 lead to a calculated $\tau_R = 1.7 \times 10^{-8}$ sec for the C-2 nucleus of the histidine. Table IV collects some calculated values of T_{1R}/T_1, T_{2R}/T_2, and NOE when τ_G is varied while τ_R is taken as 1.7×10^{-8} sec. These calculations show that even internal motion of the imidazole ring slower than a factor of 2 or 3 relative to the tumbling of the entire protein in solution would be clearly manifest in the observed relaxation parameters. We observe no such effects, and therefore conclude that, both at pH 5.8 and 8.2, the histidine side chain is locked within the catalytic triad of α-lytic protease and experiences no internal motion independent of the tumbling of the protein itself in solution.

At pH 3.3, two of the three observed resonances have line widths (~30 Hz) corresponding to a C-2 carbon with the same correlation time (1.7×10^{-8} sec) observed for the single resonance above pH 5. This suggests that these two signals arise from a carbon nucleus in an immobile histidine ring. The third signal ($\nu_{1/2} \leq 12$ Hz) represents a histidine ring with much greater mobility. The shorter correlation time, together with a chemical shift (−134.05 ppm) almost identical with that of the C-2 histidine carbons of denatured β-lytic protease, suggests that this signal arises from a histidine which is exposed to solvent and is relatively unrestrained in its movement.

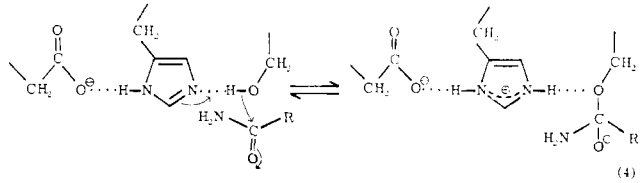
Catalytic Mechanism of Serine Proteases. The precisely oriented array of three functional groups from three amino acid residues (a hydroxyl group of serine, an imidazole ring of histidine, and a carboxylate anion of aspartic acid) has been shown to be essential for the catalytic activity of serine proteases by both chemical and physical data. From the first discovery of the buried aspartate carboxylate and the abundant proposal of a "charge relay system" consisting of the hydrogen bonded network of carboxylate, imidazole, and hydroxyl, attempts have been made to account for catalysis by increased nucleophilicity of the serine hydroxyl by partial transfer of the negative charge from the carboxylate anion to the serine oxygen in the ground state (Blow *et al.*, 1969). However, as there is no evidence for an abnormally low pK_a for the serine hydroxyl group, the degree of this charge transfer in the ground state should be minimal.

TABLE IV: Effect of τ_G on T_1, T_2, and NOE.[a]

τ_G (sec)	T_{1R}/T_1	T_{2R}/T_2	NOE
$\gg 1 \times 10^{-7}$	1.00	1.00	1.18
1×10^{-7}	1.04	0.94	1.19
1×10^{-8}	1.33	0.72	1.24
1×10^{-9}	1.09	0.45	2.05
1×10^{-10}	0.39	0.28	1.86
1×10^{-11}	0.26	0.25	1.29
$\ll 1 \times 10^{-11}$	0.25	0.25	1.18

[a] Data calculated according to Doddrell *et al.* (1972) for a carbon bonded to a single hydrogen and having a τ_R of 1.7×10^{-8} sec and only one degree of internal motion (rotation about an axis perpendicular to the C–H bond, see text).

Both the formation of the isolable acyl-enzyme intermediate from substrate and enzyme and its subsequent hydrolysis by reaction with a molecule of water probably proceed by way of a tetrahedral intermediate. Considerable evidence for obligatory intervention of such a tetrahedral species in the formation of the acyl-enzyme intermediate has been presented (Fersht and Requena, 1971, and others). Based on the commonly accepted relative pK_a values for the carboxylic acid group and imidazole ring (pK_a His ~ 6.7, pK_a Asp ~ 4.5; pK_a His > pK_a Asp) the formation of this tetrahedral intermediate must involve some charge separation. Positive character will develop on the imidazole ring as negative character builds up on the carbonyl oxygen upon formation of the new carbon–oxygen bond between the carbonyl carbon of the substrate and the serine oxygen of the enzyme. At the same time, the proton of the serine hydroxyl will be transferred to N-1 of the imidazole ring without (given that pK_a His > pK_a Asp) concomitant transfer of the other proton at N-3 to the adjacent carboxylate anion. Accordingly, the imidazole ring will become positively charged (eq 4). Though some

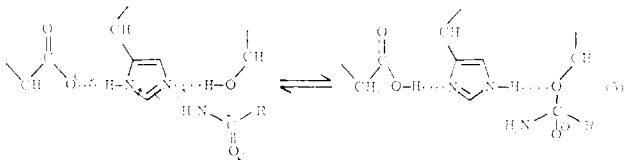


(4)

recent discussions of the formation of the tetrahedral intermediate (Robertus *et al.*, 1972) have finessed the need for such charge separation by structures which show the relevant protons ambiguously bonded to both nitrogen and oxygen, its occurrence seems logically inescapable as long as the aspartic acid carboxyl is a significantly stronger acid ($pK_a < 4$) than the imidazolium ring of the histidine (pK_a = 6.7).

Such charge separation in the hydrophobic, nonpolar region of the active site should raise the energy required to form the tetrahedral intermediate and as a result seems a teleologically unsatisfactory aspect of catalysis.

On the other hand, if, as the results of this work show, the aspartic acid carboxyl is actually a weaker acid (pK_a = 6.7) than the imidazolium ring of the histidine (pK_a < 4), formation of the tetrahedral intermediate will be accompanied by proton transfer from the serine hydroxyl to N-1 of the histidine which will be concerted with transfer of the proton at N-3 of the histidine to the carboxylate anion of aspartate. Charge separation will not be required. The negative charge on the carboxylate anion will be transferred fully to the carbonyl oxygen of the substrate and, from there, quite probably to other groups on the enzyme such as the backbone NH groups of Ser 195 and Gly 193 in chymotrypsin (Robertus *et al.*, 1972) by hydrogen bond formation to the oxyanion of the tetrahedral intermediate (eq 5).



Thus, the carboxylate anion serves not just as a passive residue whose sole role is to keep the imidazole ring properly oriented, but rather as the ultimate base which accepts the proton taken from the serine hydroxyl on formation of the tetrahedral intermediate. The imidazole ring serves a dual function. First, it insulates the carboxylic acid from water and thereby ensures it a hydrophobic environment such that its pK_a is raised to 6.7; this makes the conjugate carboxylate anion unusually basic. Second, the imidazole ring, by virtue of being a bidentate acid–base, provides a relay for net transfer of a proton from the serine hydroxyl to the buried, basic carboxylate anion. The precise positioning and immobility of the imidazole ring demonstrated by the relaxation and NOE measurements are undoubtedly important in facilitating this proton transfer (Wang, 1970).

As the pK_a of the carboxylic acid group of 6.7 is so close to that of a normal imidazolium cation of histidine, one can ask what purpose is served in having a catalytic triad of Asp-His-Ser rather than a simpler diad of His-Ser. In the case of the His-Ser diad, the imidazole ring will be the ultimate acceptor of the proton which, for a nucleophilic addition to a carbonyl group, must inevitably lead to charge separation. The Asp-His-Ser triad, in contrast, can smoothly transfer a negative charge of the carboxylate anion through the imidazole ring and serine hydroxyl to the carbonyl oxygen of the substrate; no charge separation is required. Such factors as destabilization of the carboxylate anion of Asp 102 in the Michaelis complex (as evidenced by the unusually high pK_a of 6.7 we observe for Asp 102), in addition to stabilization of the tetrahedral intermediate by hydrogen bonding and the lack of charge separation during its formation, probably account in significant measure for the catalytic efficiency of the serine proteases.

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Estimate of Minimal Distance between Rapidly Exchanging Zinc and Nucleotide Binding Sites in Liver Alcohol Dehydrogenase†

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ABSTRACT: A hybrid horse liver alcohol dehydrogenase (Young, M., and Wang, J. (1971), *J. Biol. Chem. 246*, 2815) prepared by substituting cobalt(II) for the rapidly exchanging ("catalytic") zinc atoms shows an absorption maximum at 650 mμ and an enhanced absorption in the wavelength range from 300 to 450 mμ. The appreciable spectral overlap between the absorption spectrum of this hybrid enzyme and the fluorescence emission of binary complexes with NADH, thionicotinamide NADH, and Rose Bengal results in energy transfer from the bound ligand to the cobalt in the hybrid enzyme. Calculations based upon the Förster equation require a distance of at least 19 Å between the nicotinamide ring and the cobalt binding sites. These data make it unlikely that the readily exchanging zincs of liver alcohol dehydrogenase are directly involved in the catalytic process.

The role of zinc ions in the catalytic mechanism of horse liver alcohol dehydrogenase has been investigated by numerous workers (Plane and Theorell, 1961; Drum and Vallee, 1970a,b; Iweibo and Weiner, 1972). Weiner (1969) and Mildvan and Weiner (1969) have probed the active-site region of the dehydrogenase with a spin-labeled (nitroxyl) analog of ADP-ribose[1] which binds strongly to the NADH binding site. By studying the effect of the unpaired electron on the nitroxyl radical upon the proton resonance of ethanol, they were able to estimate the distance between the substrate alcohol and the ribosidic bond to the pyridine nitrogen of NADH. Proposed mechanisms for dehydrogenase activity have assumed that the zinc atoms at the active site are in close proximity to both nucleotide and ethanol; it has been postulated (Theorell and McKinley McKee, 1961) that the zinc atom binds portions of both substrate and cofactor as ligands. Recent data of Iweibo and Weiner (1972), however, have indicated that the strength of binding of NADH and NAD^+ is not changed when the dehydrogenase is stripped of all zinc. There are four zinc atoms per dehydrogenase molecule, two readily exchangeable and two only slowly replaceable (Drum *et al.*, 1969). The loss of the rapidly exchanging zinc atoms is accompanied by a proportionate decrease in enzyme activity, and so it has been assumed that these "catalytic" zincs are situated near the active site. Iweibo and Weiner's (1972) finding that loss of *both* "catalytic" zinc and slowly exchanging structural zinc is *not* crucial for the binding of NADH suggests that even the "catalytic" zinc may also function to maintain appropriate structure at the active site.

Young and Wang (1971) have reported production of a hybrid alcohol dehydrogenase enzyme in which the two rapidly exchanging zinc ions are replaced by cobalt atoms. Use of this hybrid enzyme with cobalt in place of zinc together with an appropriate fluorescent donor (NADH or an NADH analog) held promise of estimating distances between the cobalt (or zinc) ions and the NADH moiety. Such distance calculations based upon use of the Förster equation have been extensively utilized by Latt *et al.* (1972) who studied cobalt quenching of fluorescence of synthetic carboxypeptidase substrates when cobalt is substituted for the native zinc in this metalloenzyme. It was hoped similarly that estimates of the distance between cobalt and the NADH molecule, in conjunction with the distance estimate of Mildvan and Weiner (1969), could be used to determine whether or not the cobalt

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[1] Abbreviations used are: NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; K_dK_i, dissociation constant for the enzyme–inhibitor complex; sNADH, thionicotinamide analog of NADH; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid; ADP-ribose, adenosine diphosphate ribose; K_m, apparent Michaelis constant for substrate under discussion.