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# Communication

## Ionization Behavior of the Histidine Residue in the Catalytic Triad of Serine Proteases

MECHANISTIC IMPLICATIONS*

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MICHAEL W. HUNKAPILLER,‡ STEPHEN H. SMALLCOMBE, DON R. WHITAKER,§ AND JOHN H. RICHARDS

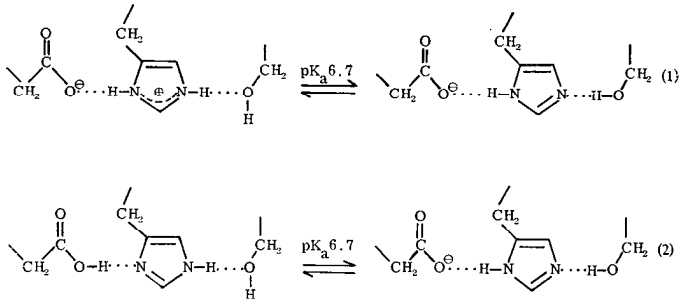
*From the Church Laboratory of Chemical Biology, California Institute of Technology, Pasadena, California 91109*

### SUMMARY

**$\alpha$-Lytic protease is a homologue of the mammalian serine proteases such as trypsin, chymotrypsin, and elastase, and its single histidine residue belongs to the Asp-His-Ser catalytic triad. This single histidine residue has been selectively enriched in the C-2 carbon with $^{13}C$. Magnetic resonance studies of the chemical shift and coupling constant ($^1J_{CH}$) behavior of this nucleus as a function of pH suggest that the imidazole ring is neutral above pH 5 and therefore that the group which is known to ionize with $pK_a$ near 6.7 must be the aspartic acid residue.**

**Implications of these new $pK_a$ assignments for the catalytic mechanism of serine proteases are discussed and include the absence of any need to separate charge during catalysis. The histidine residue plays two roles. (*a*) It insulates the aspartic acid from an aqueous environment and accordingly raises its $pK_a$. (*b*) It serves as a bidentate base to accept a proton from the serine at one of its nitrogens and concertedly transfer a proton from its other nitrogen to the buried carboxylate anion during formation of the tetrahedral intermediate.**

The three-dimensional structure of chymotrypsin, as determined by x-ray diffraction techniques (1), first revealed the presence of a buried aspartic acid residue (2) which participates in a triad of residues (Asp-102-His-57-Ser-195) which was responsible for catalysis. Many studies have implicated a group (or, more properly, a complex of residues) with a $pK_a$ near 6.7 as crucial for catalytic activity, and this $pK_a$ has been commonly identified with the imidazole ring of the histidine in the catalytic triad (3–5). 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 two situations (see Equations 1 and 2) could describe the available evidence. Since such a difference in microscopic ionization behavior of the aspartic acid and histidine residues would have major significance for the actual charge transfers and hydrogen movements that occur




during catalysis, we sought to determine the state of ionization of the imidazole ring as a function of pH.

The protein actually investigated was $\alpha$-lytic protease which we chose for a variety of reasons. (*a*) $\alpha$-Lytic protease, in binding and catalysis, displays the characteristics of other serine proteases—particularly of elastase—and can, therefore, serve as a paradigm of these enzymes (6–11). (*b*) It possesses only a single histidine residue which belongs to the catalytic triad and whose properties can accordingly be unambiguously studied. (*c*) It exhibits remarkable stability toward denaturation and autolysis even under conditions of pH and temperature where catalytic activity is maximal.

### EXPERIMENTAL PROCEDURE

L-[2-$^{13}C$]Histidine (90% enrichment) synthesized by published procedures (12) was incorporated into $\alpha$-lytic protease by Myxobacter 495 (9). The resulting enrichment in the isolated, purified protein was judged to be ~60%. That all label was still present in the single histidine residue was confirmed by parallel experiments using L-[2-$^{14}C$]histidine. Hydrolysis of the resulting, purified protein showed significant $^{14}C$ activity only in histidine. All other amino acids had less than 1% the activity of the histidine, although in this experiment the added $^{14}C$ label had been diluted to ~50% its original concentration.

Fig. 1 shows typical natural abundance and enriched proton-noise decoupled CMR[1] spectra of $\alpha$-lytic protease at pH 5.7. The spectra are of unbuffered (0.2 M KCl) aqueous solutions with 5 to 6 mM concentration of protein. The spectra were recorded on a Varian XL-100-15 NMR spectrometer operating at 25.17 MHz in the Fourier transform mode. The absorption at −134.6 ppm (Fig. 1) relative to external tetramethylsilane, which appears in the spectrum of the enriched enzyme but is absent in the spectrum of the natural enzyme, is assigned to the C-2 carbon of the single histidine. Coupling between the C-2 of histidine and its attached proton ($^1J_{CH}$) was also observed in coupled spectra such as those in Fig. 2. Tables I and II collect the results of these and other relevant observations. ($\beta$-Lytic protease, which contains 8 histidine residues, is also produced by Myxobacter 495 and, when denatured, provides a convenient model for histidine in a random coil polypeptide.)

### DISCUSSION

The data of Table I show for protonated imidazole and its derivatives a chemical shift range of −133.13 to −135.52 ppm

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§ Department of Biochemistry, University of Ottawa, Ottawa, Canada.

[1] The abbreviation used is: CMR, carbon magnetic resonance.

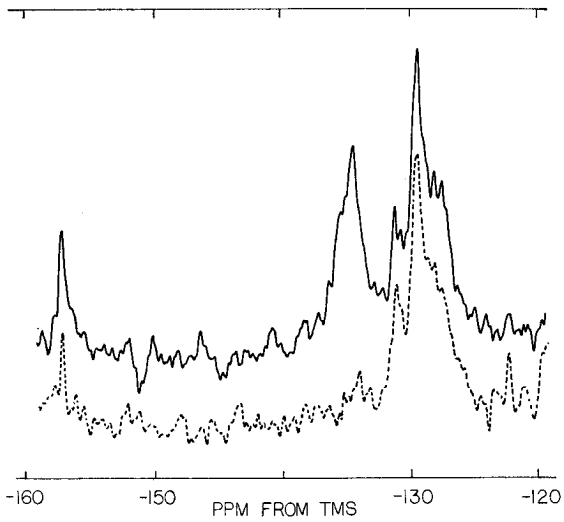



FIG. 1. Proton-noise decoupled CMR spectra of $\alpha$-lytic protease. - - -, natural abundance $^{13}C$; ——, $^{13}C$-enriched at histidine C-2. Enzyme (6 mM), KCl (0.2 M), 34°, pH 5.75. Each spectrum represents 200,000 transients at 0.15-s acquisition time, 2,000-Hz sweep width, 90° pulse.

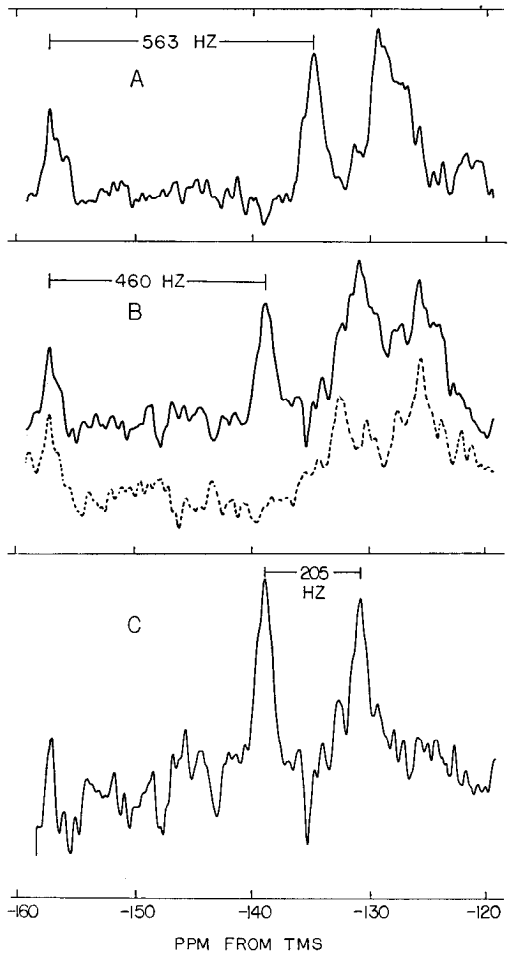



FIG. 2. Measurement of $^1J_{CH}$ for histidine C-2 in $\alpha$-lytic protease at pH 5.98. *A*, proton decoupled CMR spectrum, $^{13}C$-enriched, 50,000 transients. *B*, ——, proton coupled CMR spectrum, same sample as in *A*, 250,000 transients; - - -, proton coupled CMR spectrum, natural abundance $^{13}C$, 250,000 transients. *C*, difference spectrum obtained by computer subtraction of the natural abundance spectrum (*dashed line*) from $^{13}C$-enriched spectrum (*solid line*) 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.

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 TMS[b]) | | $^1J_{CH}$ (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[c] (denatured) | −134.09 | −136.67 | 218 | 206 |

[a] Measured for 1 to 2 M aqueous solutions unless otherwise indicated.
[b] TMS, tetramethylsilane.
[c] 1 to 2 mM solution in 0.2 M KCl.

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 TMS[a]) | $^1J_{CH}$ (Hz ± 3) |
|---|---|---|
| 8.2 | −136.95 | 205 |
| 5.2 | −134.57 | 205[b] |
| 3.3 | −134.81 | 208 |
| | −134.05 | 222 |
| | −132.46 | 218 |

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

and for neutral imidazole a chemical shift range of −134.49 to −139.45 ppm. In contrast to this varied response of chemical shift to the state of ionization, the coupling constant, $^1J_{CH}$, has a narrow range of values in both the cationic (220 ± 2 Hz) and neutral (207 ± 2 Hz) states. We take these results to indicate that the coupling constant reflects reliably the true state of ionization of imidazole and its derivatives while the chemical shift may be significantly affected by other factors.

We observe that $^1J_{CH}$ for C-2 of the histidine residue of $\alpha$-lytic protease has a value characteristic of a *neutral* imidazole ring from pH 5.2 to 8.2 ($^1J_{CH}$ 205 Hz). 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 (Fig. 3). One, which disappears below pH 3, has the same chemical shift (−134.8 ppm), $^1J_{CH}$ (208 Hz), and linewidth (30 Hz) as the single resonance observed at pH 5.2. This resonance probably represents a neutral histidine residue. 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 linewidth (15 Hz) of this signal is considerably narrower than that of the other two (indicating considerably more side chain mobility), and we assign this resonance to a protonated histidine that has been ejected from its normal, partially buried position as a member of the catalytic triad into solution. 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 linewidth (30 Hz), suggests that it represents the protonated histidine near its normal position in the catalytic triad.

In summary, only below pH 4 does the histidine exist in a protonated state. Around pH 5 to 6, the imidazole ring of

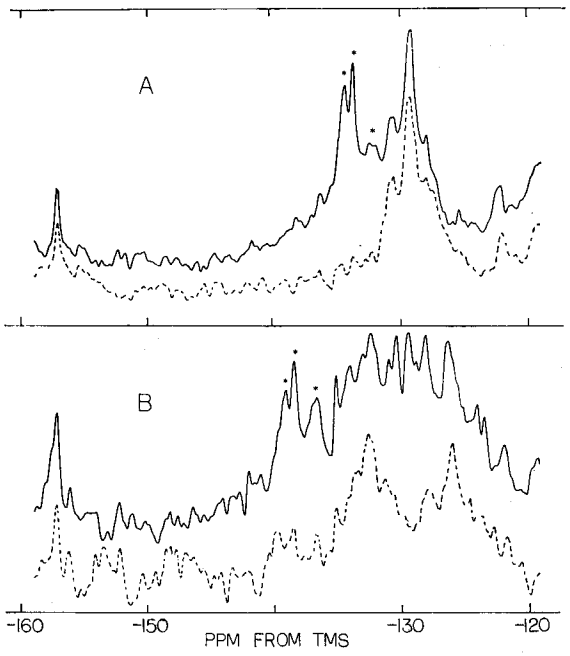
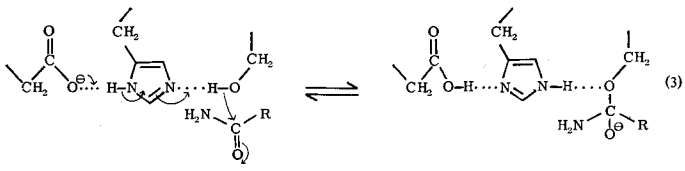



FIG. 3. CMR spectra of $\alpha$-lytic protease at pH 3.25. ——, $^{13}C$-enriched; – – –, natural abundance $^{13}C$. *A*, proton-noise decoupled; *B*, proton coupled. The resonances assigned to C-2 of the histidine residue are marked by an *asterisk*. Each spectrum represents 250,000 transients at 0.15-s acquisition time.

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 (Equation 1), but rather a neutral system of carboxylic acid-imidazole (Equation 2). These results imply that, during catalysis, there is a transfer of negative charge from the carboxylate anion of the aspartate to the carbonyl oxygen of the bond being hydrolyzed. Protons move concertedly from serine to histidine and from histidine to aspartate to accommodate this charge transfer (see Equation 3). The alternative situation which follows if protonation of the catalytic triad occurs according to Equation 1 would require charge separation during catalysis. Such charge separation in



(3)

the hydrophobic, nonpolar region of the active site (2) should increase the energy necessary to form the tetrahedral intermediate (thus making its formation more difficult) and seems a teleologically unsatisfactory aspect of catalysis.

Correct recognition of the ionization behavior of these residues avoids charge separation in mechanistic proposals of serine protease catalysis, and the reasons nature has evolved the catalytic triad of Asp-His-Ser thereby become clear. The carboxylate anion serves as the ultimate base which accepts the proton liberated from Ser-195 on formation of the tetrahedral intermediate. If there were only a His-Ser diad, the histidine would have to be the ultimate proton acceptor, and, for reactions such as amide hydrolysis which require nucleophilic attack on the carbonyl group, this would lead *pari passu* to charge separation. 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 in turn 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 protons from the serine hydroxyl to the buried, basic carboxylate anion. 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 (13) and the lack of charge separation during its formation, probably accounts in significant measure for the catalytic efficiency of the serine proteases.

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