Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7323–7326, December 1981
Biochemistry

Catalytic mechanism of serine proteases: Reexamination of the pH dependence of the histidyl $^1J_{^{13}C2\text{-}H}$ coupling constant in the catalytic triad of α-lytic protease*

(¹³C NMR/enzyme mechanisms/biosynthetic isotopic enrichment/histidine auxotroph/charge-relay system)

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Contributed by John D. Roberts, August 10, 1981

ABSTRACT L-Histidine, 90% ^{13}C enriched at the C2 position, was incorporated into the catalytic triad of α-lytic protease (EC 3.4.21.12) with the aid of a histidine-requiring mutant of *Lysobacter enzymogenes* (ATC 29487), and the pH dependence of the coupling constant between this carbon atom and its directly bonded proton was reinvestigated. The high degree of specific ^{13}C isotopic enrichment attainable with the auxotroph permits direct observation and measurement of this coupling constant in proton-coupled ^{13}C NMR spectra at 67.89 MHz and at 15.1 MHz. In contrast to the earlier study, the present results indicate that this coupling constant does respond to a microscopic ionization with pK_a near 7.0; moreover, the magnitude of the values of $^1J_{C\text{-}H}$ observed are in accord with those expected for titration of the histidyl residue. We conclude that the original measurement must be in error and that this coupling constant now also supports a histidyl residue that titrates more or less normally as a component of the catalytic triad of serine proteases.

A "catalytic triad" comprised of the side-chain functional groups of aspartic acid, histidine, and serine has thus far proved to be an invariant feature of the active sites of serine proteinases as demonstrated by x-ray diffraction studies (1–6). The ubiquity and diversity of individual enzymes belonging to this class suggests that this array of Asp-His-Ser residues possesses special catalytic properties. The precise mode of operation of this triad in serine protease-catalyzed hydrolysis of amides and esters is, therefore, of considerable interest.

A prerequisite to the understanding of the effectiveness of this triad is a knowledge of the ionization behavior of its component functional groups, and this has been a controversial issue. A histidyl residue is essential for activity (7–10), and because the activities of serine proteinases increase with pH in a manner indicative of the titration of a single group having a pK_a ≈7.0 (11), this ionization was originally assumed to represent that of the particular histidyl residue. However, Hunkapiller *et al.* (12) proposed that this pK_a of 7.0 should instead be assigned to the aspartic acid residue and that the histidyl residue should be assigned a pK_a of less than 4.0. The experimental basis for this proposal was a determination that the coupling constant between C2 of the histidyl residue in the catalytic triad of α-lytic protease and its directly bonded proton was independent of pH over the range 4.0–8.0 and indicative of a neutral imidazole ring. The result of this effective reversal of normal pK_a assignments is to make the aspartic acid carboxylate the ultimate charge donor in the operation of the so-called "charge-relay" mechanism (1, 12) of attack on the peptide bond.

The hypothesis that histidyl residues in the catalytic triads of serine proteases are abnormally weak bases, whereas the corresponding aspartic acid residues are abnormally weak acids, has received considerable support, both experimental (13–18) and theoretical (19–23). There are, however, other experimental results (24–28) that indicate more normal ionization behavior; at one time, substantial controversy on this point existed. Recent ^{15}N (29) and ^1H NMR (30–32) studies strongly indicate that histidyl residues at the catalytic site titrate more or less normally. Nevertheless, the experimental data originally supporting the pK_a-reversal hypothesis remain to be reconciled with these studies. Especially troublesome are the measurements of the histidyl $^1J_{^{13}C2\text{-}H}$ coupling constant for α-lytic protease (12) because this result is difficult to attribute to anything but a histidyl residue with an abnormally low pK_a.

The reported measurements of $^1J_{^{13}C2\text{-}H}$ are not free of difficulties. A major problem is that the difference in magnitude of this coupling constant between the protonated (≈218 Hz) and neutral (≈208 Hz) forms of the imidazole ring is small, and its measurement in α-lytic protease was hampered by large linewidths and by background natural-abundance resonances that obscured one line of the doublet. Therefore, determination of the coupling required measurement of $1/2\,J$ or the taking of difference spectra. Indeed, whether this measurement could be made with sufficient precision under these circumstances has been questioned (26, 33).

Improved NMR instrumentation operating at higher magnetic field offers the possibility of enhancing the accuracy of the measurements because, at higher fields, interference from background natural-abundance signals should be substantially reduced. Also, a histidine-requiring mutant of *Lysobacter enzymogenes* is now available which allows one to achieve a higher specific ^{13}C enrichment and, thus, to obtain improved signal detection and resolution. In view of these improved prospects for measuring this coupling constant and the difficulties associated with the earlier study, we report here a reexamination of its pH dependence in α-lytic protease.

MATERIALS AND METHODS

L-Histidine, selectively enriched with ^{13}C at C2 was obtained from Isotope Labelling (Whipp, NJ), or KOR Isotopes, (Cambridge, MA), and was synthesized from L-2,5-diamino-4-ketovalertic acid and $KS^{13}CN$ as described by Ashley and Harrington (34) and Heath *et al.* (35). Each preparation was judged to be roughly equivalent in regard to purity and specific ^{13}C enrichment (≈92%) by ^{13}C NMR spectroscopy. Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide was synthesized as described by Hunkapiller *et al.* (36) and used to assay the activity of the enzyme.

The ^{13}C-labeled histidyl-α-lytic-protease was prepared and purified by culturing a histidine-requiring mutant of *L. enzymogenes* using the previously described procedures (12, 29). The

* Presented in part at the *Ninth International Conference on Magnetic Resonance in Biological Systems*, Bendor, France, September 1–6, 1980.

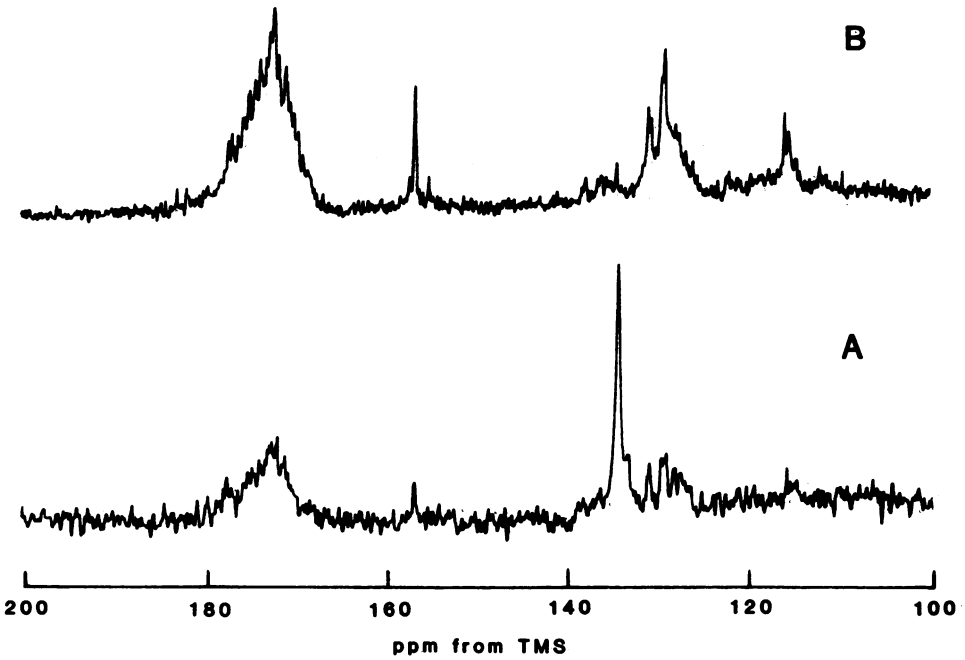

FIG. 1. Proton-decoupled 67.89-MHz ^{13}C NMR spectra of α-lytic protease. (*A*) [2-^{13}C]Histidyl-enriched α-lytic protease (≈3 mM at pH 4.7; 6400 scans with a recycle time of 0.84 sec). (*B*) Natural-abundance α-lytic protease (≈8 mM at pH 6.0; 46,000 with a recycle time of 2 sec).

peptidase activity of α-lytic protease was assayed against Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide (4×10^{-4}M in 0.05 M Tris buffer, pH 8.75, at 25°C). Based on $A_{278}^{1\%} = 8.9$, purified preparations of α-lytic protease used in these NMR studies exhibited k_{cat}/K_m values of 2.0×10^3 M^{-1} s^{-1} as compared to a value of 1.5×10^3 $M^{-1}s^{-1}$ reported previously (36).

^{13}C NMR spectra were recorded at 67.89 MHz on a Bruker HX-270 spectrometer and at 15.08 MHz on a Bruker WP-60 spectrometer; 10-mm probes were used with both instruments. The NMR samples were 1–5 mM in α-lytic protease and were

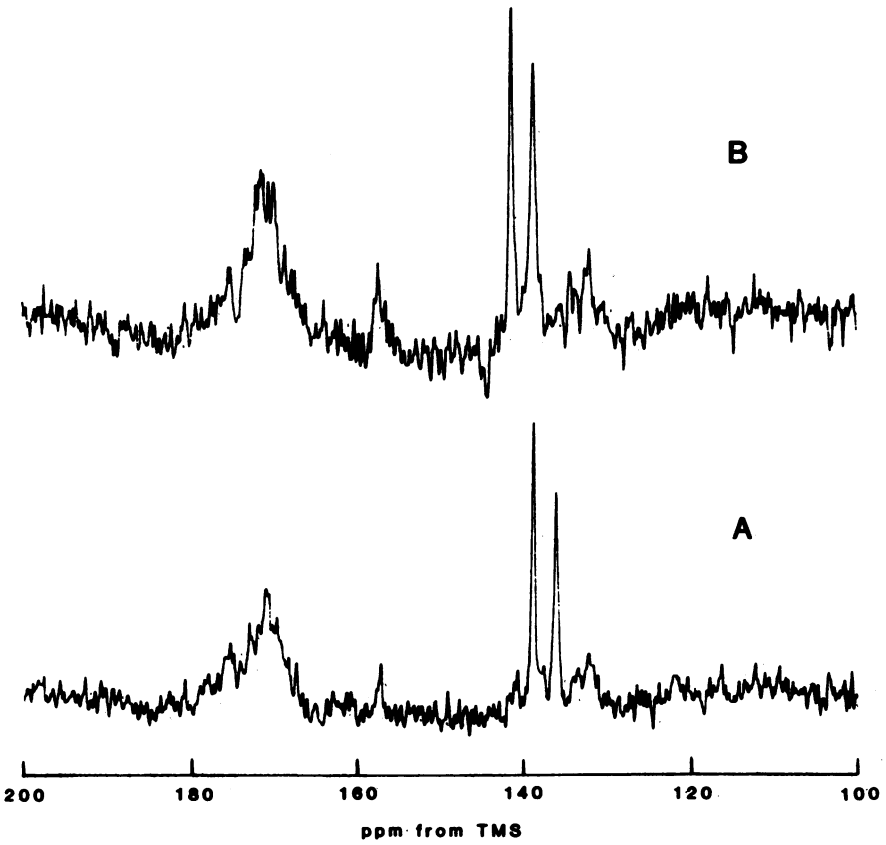

FIG. 2. Proton-coupled 67.89-MHz ^{13}C NMR spectra of [2-^{13}C]histidyl-enriched α-lytic protease. (*A*) Enzyme (1.5 mM) at pH 5.54 (25,300 scans with a recycle time of 0.84 sec). (*B*) Enzyme (1.3 mM) at pH 8.24 (38,500 scans with a recycle time of 0.84 sec).

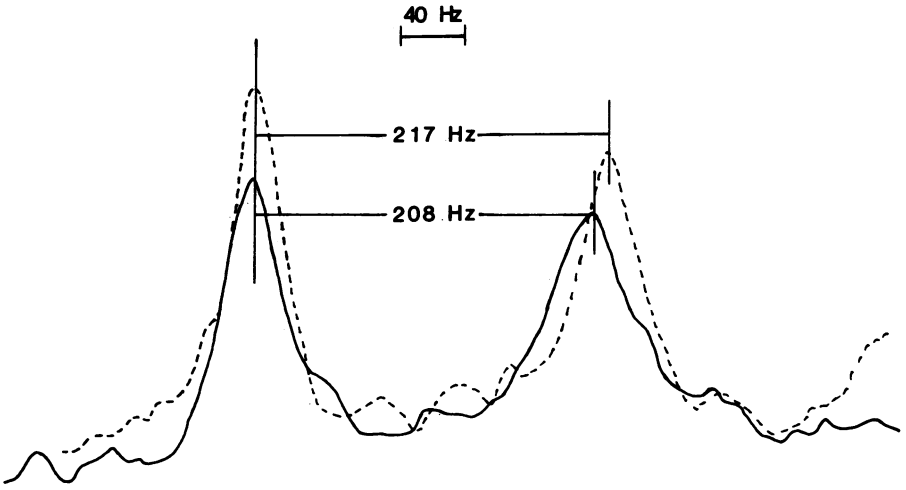

FIG. 3. Comparison of representative high and low pH doublets from 67.89-MHz ^{13}C proton-coupled spectra of [2-^{13}C]histidyl-enriched α-lytic protease. ——, Enzyme (1.34 mM) at pH 8.24 (38,550 scans); ----, 1.5 mM enzyme at pH 5.25 (51,960 scans).

prepared by dissolving lyophilized powders of enzyme in 0.1 M KCl. About 15% of ^2H_2O was added to provide an internal field frequency lock signal. The relatively sharp signal in ^{13}C NMR specta of α-lytic protease arising from the guanidinium carbons of the 12 arginine residues (and previously assigned a chemical shift of 157.25 ppm relative to tetramethylsilane) was used as an internal reference after its position relative to internal dioxane was verified to be the same at high and low pH. Chemical shifts are reported in ppm from tetramethylsilane.

In general, 67.89-MHz ^{13}C spectra were acquired by using a 90° radiofrequency pulse (26 μs)1, a spectral width of 16,000 Hz, and 8000 data points. The ^{13}C spectra at 15.08 MHz were acquired with a 90° pulse (21 μs), a spectral width of 4000 Hz, and 2000 data points.

The pH of the solution and the specific activity of the enzyme were checked both before and after recording each spectrum; only for those samples which exhibited no discernible change in these parameters are spectra reported here. The pH of the sample was varied by the addition of 0.25–0.5 M NaOH or HCl.

RESULTS AND DISCUSSION

Representative proton-decoupled 67.89-MHz ^{13}C NMR spectra of unlabeled α-lytic protease and of [2-^{13}C]histidyl-labeled α-lytic protease are compared in Fig. 1. The large single resonance at 135 ppm present only in the spectrum of the isotopically enriched enzyme is clearly that of the ^{13}C-labeled carbon of the histidyl residue. The pH dependence of the chemical shift of this resonance is the same as reported earlier (12). Representative proton-coupled ^{13}C NMR spectra at high and low pH are shown in Fig. 2; now both lines of the doublet are clearly resolved at high and low pH, so that $^1J_{C-H}$ can be measured directly from the peak separation. Six independent determinations of $^1J_{C-H}$ were made at pH values of 4.66, 5.25, 5.35, 5.47, 5.54, and 6.02, which gave values for $^1J_{C-H}$ of 219, 217, 219, 217, 217, and 216 Hz, respectively. Two determinations of $^1J_{C-H}$ at pH 8.24 and 8.44 gave values of 208 and 204, respectively. Either Lorentzian or parabolic interpolation of the peak positions yielded the same value for $^1J_{C-H}$. The curves in Fig. 3 for representative high and low pH doublets demonstrate that $^1J_{C-H}$ does change with pH.

In addition to the high-field ^{13}C NMR measurements at 67.89 MHz, the coupling constant was also determined by ^{13}C NMR spectroscopy at 15.1 MHz, and even at this lower magnetic field, both lines of the doublet were sufficiently resolved to allow direct measurement of the coupling. Two independent determinations of the coupling constant in both the high and low pH ranges gave effectively the same results as the measurements at 67.89 MHz.

The present results indicate that this coupling constant does respond to an ionization of the histidyl residue with a pK_a near 7.0, and the original measurements (12) must be in error. The source of this error is, at present, not clear, but possibly derives from the presence of multiple forms of the enzyme (31) at acidic pH. These forms can be resolved at 125 MHz where they are in slow exchange (R. J. Kaiser and T. G. Perkins, personal communication).

Consequently, the NMR data (^{15}N, ^{13}C, and ^1H) now support a histidyl residue which titrates more or less normally as a component of the active-site catalytic triads of serine proteases—at least for the free enzyme in solution. Other experimental or theoretical studies that support, as well as mechanistic schemes based upon, the pK_a-reversal hypothesis need reappraisal.

This work was supported by grants from the National Institutes of Health (GM-27927 and GM164221) and from Research Corporation. The high-field NMR experiments were performed at the NMR Facility for Biomolecular Research located at the F. Bitter National Magnet Laboratory (Massachusetts Institute of Technology). The NMR Facility is supported by Grant RR00995 from the Division of Research Resources of the National Institutes of Health and by National Science Foundation Contract C-670.

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