High-Resolution Nitrogen-15 Nuclear Magnetic Resonance Studies of α-Lytic Protease in Solid State. Direct Comparison of Enzyme Structure in Solution and in the Solid State†

Tai-Huang Huang,‡ William W. Bachovchin,* Robert G. Griffin, and Christopher M. Dobson

ABSTRACT: Histidine enriched in ^{15}N in the imidazole nitrogens was incorporated into the catalytic triad of α-lytic protease, and high-resolution solid-state ^{15}N NMR spectra of lyophilized enzyme powders were recorded. The lyophilized powders were prepared from aqueous solutions with pH values ranging from 4.9 to 9.3. The behavior of the ^{15}N resonances as a function of "pH" in these solid samples closely parallels that observed previously in the corresponding solution-state study, with the exception that in the powders proton exchange at His-57 is slow on the NMR time scale whereas in solutions it is fast. Thus, the ^{15}N isotropic shifts demonstrate that the N^{π}–H tautomer of His-57 predominates in powders prepared at high pH and that N^{π}(H) participates in a strong hydrogen bond, as the hydrogen-bond donor, in powders prepared at both high pH and low pH. The simplest interpretation of these results is that the active site catalytic triad structure of Asp-His-Ser is maintained in these lyophilized powders. Because Asp-102 and His-57 are sequentially separated, their interaction in these lyophilized powders suggests that the tertiary structures of α-lytic protease in the powder and in solution are very similar. The ^{15}N isotropic shifts further indicate that His-57 located within the intact triad in lyophilized enzyme powders has what can be taken as a normal "pK_a" for a histidyl residue, undergoing a transition from the protonated to the neutral state with a midpoint between pH 6.0 and 7.0.

To understand how enzymes work at the molecular level remains an important objective in biochemistry. A knowledge of molecular structure is crucial to this task because, by definition, it involves relating the catalytic properties of an enzyme to its molecular structure. For structural information, one, at present, turns to the results of X-ray diffraction studies. A protein crystal lattice is, however, a different environment from the solution state in which the catalytic properties of an enzyme are normally defined and studied (Karplus & McCammon, 1981; Johansen & Vallee, 1973). Moreover, X-ray crystallography generally provides a static picture of one conformation whereas globular proteins are increasingly being viewed as dynamic structures, able to adopt different conformations that may rapidly interconvert in solution (Karplus & McCammon, 1981; Jardetzky & Roberts, 1981; Gurd & Rothgeb, 1979). Therefore, before interpreting catalytic properties in terms of molecular structure, one must first ask how closely the crystallographically determined structure corresponds to the solution-state structure. This question is especially critical as it pertains to the juxtaposition of active site catalytic groups. Even small differences in the relative location of these groups between crystals and solutions could seriously mislead efforts to understand their catalytic roles.

Comparative studies of the crystal and solution structures of enzymes are therefore highly desirable, and much effort in this direction already has been expended (Wüthrich et al., 1982; Dobson, 1977; Johansen & Vallee, 1973). Most often, however, these studies have taken the form in which a particular structural feature of the solution-state enzyme is examined through a technique applicable to solutions and the result then compared with the X-ray structure. Valuable as these studies are, the two sets of experimental data are not in themselves directly comparable. Ideally, such comparative studies should apply the same technique to both states. This would permit a direct and, therefore more straightforward, comparison. In this work, we explore the potential of using NMR spectroscopy to gain such direct comparisons of enzyme structures in solution and solid states by recording MASS[1] ^{15}N NMR spectra of ^{15}N-labeled α-lytic protease in solid samples and comparing the results to those of a corresponding solution-state study.

The above enzyme is especially attractive for such a comparative study for several reasons. First, α-lytic protease is a serine protease, and as such, it is representative of a large and important family of enzymes whose catalytic mechanism has been, and continues to be, of much interest (Kraut, 1977; Markley, 1979; Steitz & Shulman, 1982). Moreover, X-ray diffraction studies (Blow et al., 1969; Kraut, 1977; Steitz & Shulman, 1982) have played a central role in the study of these enzymes by having identified a particular alignment of the side-chain functional groups of aspartic acid, histidine, and serine, now known as the catalytic triad or charge-relay system, as an invariant feature of their active sites (**1**). Second, ^{15}N NMR spectroscopy already has been successfully used to examine His-57[2] of α-lytic protease in the solution state

† From the Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111 (W.W.B.), the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (T.-H.H. and R.G.G.), and the Inorganic Chemistry Laboratory, Oxford University, Oxford, England (C.M.D.). *Received February 3, 1984; revised manuscript received June 14, 1984.* This work was supported by U.S. Public Health Service Grants GM-27927 (W.W.B.) and GM-23403 (R.G.G.) from the Division of General Medical Sciences, by National Institutes of Health Research Grant RR-00995, and by the National Science Foundation through its support of the Frances Bitter National Magnet Laboratory (DMR-8211416). W.W.B. is a National Institutes of Health Research Career Development Awardee (AM01122). Presented in part at the 10th International Conference on Magnetic Resonance in Biological Systems, Stanford University, Stanford, CA, Aug 29–Sept 3, 1982.

‡ Present address: Department of Physics, University of Maine, Orono, ME 04469.

[1] Abbreviation: MASS, magic angle sample spinning.

[2] The chymotrypsin numbering system is used in specifying the residues of the catalytic triad.

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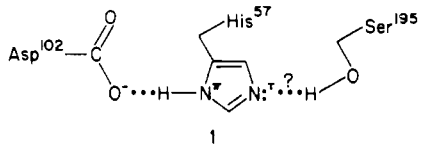

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(Bachovchin & Roberts, 1978). This study confirmed expectations that the ^{15}N chemical shifts would be very informative not only about the protonation state of His-57 but also about its tautomeric structure and its hydrogen-bond interactions and, therefore, about its immediate environment in the active site. Finally, the amino acid composition of α-lytic protease includes only a single histidine residue—that of the catalytic triad (Olson et al., 1970), which, moreover, can be specifically and efficiently enriched in ^{15}N by using the histidine-requiring mutant of *Lysobacter enzymogenes* (Bachovchin & Roberts, 1978). Problems of detection, resolution, and assignment usually encountered in NMR studies of proteins are therefore minimized.

In preparation for eventual MASS/NMR experiments on crystals, we have examined lyophilized powders of α-lytic protease prepared from solutions with pH values ranging from 4.0 to 9.3. The behavior of the ^{15}N signals from His-57 in MASS NMR spectra of these lyophilized powders and a comparison with their behavior in the corresponding spectra of the solution-state enzyme are the subjects of this paper.

Experimental Procedures

L-Histidine, selectively enriched in ^{15}N (99%) at the π-nitrogen of the imidazole ring, and DL-histidine enriched in ^{15}N (95%) at both the π- and τ-nitrogens were purchased from Isotope Labeling Corp., Whippany, NJ.

Incorporation of labeled histidine into α-lytic protease (EC 3.4.21.12) was accomplished by culturing a histidine-requiring mutant of *Lysobacter enzymogenes* (ATC29487) as described previously. The enzyme activity was measured spectrophotometrically with Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide [4×10^{-4} M in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.75, at 25 °C]. On the basis of $A_{278}^{1\%} = 8.9$, purified preparation of α-lytic protease used in these NMR studies exhibited k_{cat}/K_M values of 2.0×10^3 M^{-1} s^{-1}.

The pH of purified enzyme solutions was adjusted with 0.20–0.50 M NaOH or HCl. Prior to lyophilization, the pH of the solution and the activity of the enzyme were carefully determined. After recording MASS NMR spectra, the lyophilized powders were redissolved in distilled H_2O, and the pH of the resulting solution and the activity of the enzyme were again determined. In general, the pH measurements before and after the NMR experiments agreed to within 0.1 pH unit while those of enzyme activity agreed to within 10%.

For NMR experiments, approximately 150 mg of lyophilized protein was tightly packed into each Andrew Beam rotor of outer diameter 0.300 in. Rotational sidebands were identified by varying spinning rates between 2 and 3.5 kHz. MASS NMR experiments were performed on a home-built spectrometer operating at a field of 6.9 T. ^{15}N signals were observed at 29.82 MHz with a proton frequency of 294.2 MHz. Pulses of 90° were typically 7 μs for ^1H and 8.5 μs for ^{15}N. All spectra were obtained by cross-polarization techniques, and a 180° pulse was inserted at a time τ_r after the mixing period, where $\nu_r = \tau_r^{-1}$ is the spinning frequency. Hahm echo is thus formed at $2\tau_r$ at which time data acquisition commences. In addition, we have found that the optimal mixing times for the protonated and nonprotonated histidine nitrogens are very different, being 0.3 ms for the protonated nitrogens and 2 ms for the nonprotonated nitrogens. For our experiments, a mixing time of 1.5 ms was used. Because of the difference in cross-polarization efficiency, the intensity ratio of the protonated and nonprotonated ^{15}N is not simply proportional to the population ratio of the two species. However, intensity measurements of the same resonance in different spectra recorded under identical conditions may be compared. To avoid heating due to high-power decoupling, samples were cooled with constant air flow at room temperature. The pulse recycling time was 5 s, and it took 20 h to obtain each spectrum. Isotropic chemical shifts were measured in ppm relative to external powdered $(^{15}NH_4)_2SO_4$ and reported here in ppm from 1 M $H^{15}NO_3$ in D_2O.

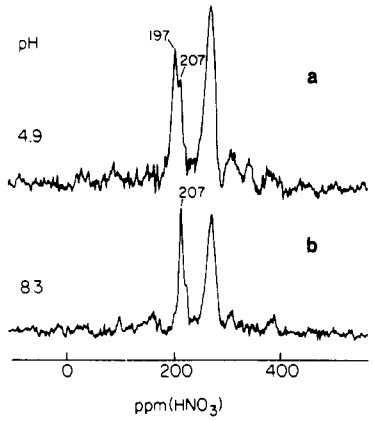


FIGURE 1: MASS ^{15}N NMR spectra of lyophilized powders of α-lytic protease labeled specifically with ^{15}N at the π-position ($N^{\delta 1}$) of His-57.

Results

MASS ^{15}N NMR Spectra of $^{15}N^{\pi}$-Labeled Histidyl α-Lytic Protease. Figure 1 shows representative MASS NMR spectra of lyophilized powders of α-lytic protease labeled specifically with ^{15}N at the π-position of His-57. Two major resonance lines appear in these spectra. The one at higher field (~255 ppm) arises from the approximately 200 amide nitrogens at the natural abundance level of ^{15}N in the peptide backbone. The lower field resonance is that of the labeled π-nitrogen in the histidyl residue of the catalytic triad.

The $^{15}N^{\pi}$ resonance exhibits a "pH dependence" in these lyophilized powders in the sense that its isotropic shift depends on the pH of the solution from which the powder is prepared. In the powder prepared from a solution at pH 4.9, the isotropic shift is 197 ppm (Figure 1a), whereas in the powder prepared from a solution at pH 8.3 the isotropic shift is 207 ppm (Figure 1b). This 10 ppm change, as will be demonstrated later, reflects a change in the protonation state of the imidazole ring. The resonance at 197 ppm arises from the protonated imidazolium ion, which predominates in the "pH 4.9" powder, whereas the resonance at 207 ppm arises from the neutral form, which predominates in the "pH 8.3" powder. The presence of both signals in MASS NMR spectra of powders at intermediate "pH" (spectra not shown) demonstrates that these two forms are in slow exchange on the NMR timescale.

MASS ^{15}N NMR Spectra of $^{15}N^{\pi}$,$^{15}N^{\tau}$-Labeled α-Lytic Protease. The behavior of the N^{τ} signal was determined by recording MASS NMR spectra of α-lytic protease labeled with ^{15}N at both nitrogens of the imidazole ring. Assignment of the N^{τ} resonance was made by comparing these spectra with those of the singly labeled enzyme. At "pH 5.1", the N^{τ} resonance occurs at 208 ppm where it is just barely resolved from the N^{π} resonance (Figure 2a). However, in a "pH 9.3" powder, N^{τ} resonates at 143 ± 5 ppm (Figure 2c). Thus, in contrast to N^{π}, which exhibits a small (10 ± 2 ppm) upfield chemical shift change when the imidazole ring of His-57 de-

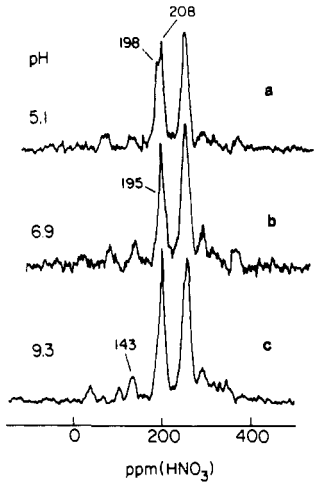


FIGURE 2: MASS ^{15}N NMR spectra of lyophilized powders of α-lytic protease labeled with ^{15}N at both the π-position ($N^{\delta 1}$) and τ-position ($N^{\epsilon 2}$) of His-57.

protonates, N^{τ} exhibits a large (66 ± 5 ppm) downfield chemical shift change moving from 208 to 143 ppm. Again, these two forms are in slow exchange.

The N^{τ} resonance at 143 ppm in the "pH 9.3" powder is very weak relative to the N^{τ} resonance at 208 ppm in the "pH 5.1" powder or relative to the N^{π} resonance in either the high-pH powder or low-pH powder. Its reduced relative intensity reflects the reduced relative efficiency with which this nonprotonated nitrogen undergoes cross-polarization as discussed under Experimental Procedures. The other relatively weak signals in these spectra are rotational sidebands as was verified by varying the spinning rates.

Comparison of Solution- and Solid-State Results. Figure 3 compares the results of the present solid-state study with those of the earlier solution-state study. Inspection of Figure 3 immediately reveals that the behavior of both the N^{π} and N^{τ} resonances of His-57 in solid samples parallels that previously observed in solution samples to a remarkable degree. Specifically, on going from pH 5.0 to 9.0, N^{π} exhibits a *small upfield* chemical shift change of 10 ± 2 ppm in the solid compared to 8 ppm in solution, while N^{τ} exhibits a *large downfield* chemical shift change of 65 ± 3 ppm in the solid compare to 66 ± 1 ppm in solution. The major difference between the solution and solid samples in in the rate at which His-57 undergoes acid–base exchange. In solution, this exchange process is fast on the NMR time scale while in the solid it is slow. Thus, in solution each nitrogen gives rise to only a single resonance, the chemical shift of which represents a weighted average of the protonated and free-base forms of His-57 present. In the solid, these two forms are separately observed. It should also be noted that the solid-state isotropic shifts do not differ greatly from the solution-state chemical shifts. Apparently, the physical state of the protein has little or no effect on the ^{15}N resonance frequencies.

Discussion

The side-chain imidazole group of histidine is often found associated with functionally important structures in proteins. NMR studies of solutions of histidine (Blomberg et al., 1977), of imidazole derivatives (Roberts et al. 1972; Shuster & Roberts, 1979), and of His-57 in α-lytic protease (Bachovchin & Roberts, 1978) have demonstrated that ^{15}N NMR holds a significant advantage over proton or carbon NMR in studying these residues; in particular, ^{15}N chemical shifts are very sensitive not only to the state of protonation of the imidazole ring but also to hydrogen-bonding interactions and to tautomeric structure. This latter information is particularly valuable as its relays information about the immediate environment of the imidazole group in the protein.

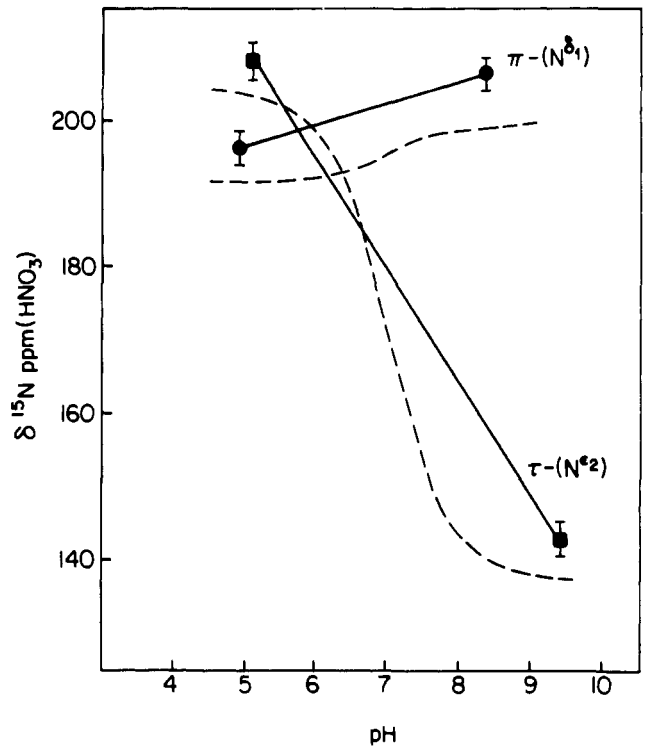
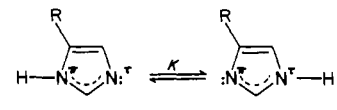


FIGURE 3: pH dependence of the ^{15}N shifts of the imidazole ring nitrogens of His-57 in α-lytic protease in solutions (– –) and in lyophilized powders (—).

We have recently shown that high-resolution ^{15}N NMR spectra can be obtained from solid samples (lyophilized powders) of imidazole and histidine and that the ^{15}N isotropic shifts so obtained respond to, and thus provide information about, the same processes as in solution (Munowitz et al., 1982). The previous study revealed that the "pH-dependent" behavior of both histidine and imidazole in lyophilized powders closely parallels their behavior in solution, with the exception that in the powders the acid–base and tautomeric exchange processes are slow on the NMR time scale whereas in solution they are fast. This study further demonstrated that the solution parameters, pH and pK_a, retained meaning for the powders in the sense that the conjugate acid/conjugate base ratio found in a solution, as governed by pH and pK_a, was also found in the powder prepared from that solution.

In the present work we have extended these studies to His-57 in lyophilized powders of α-lytic protease. We were especially interested in determining whether the active-site triad structure would remain intact and in determining whether removal of water would alter the protonation behavior of His-57 within the triad.

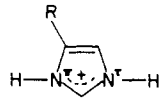
Using the ^{15}N chemical shifts for evaluating the protonation state of His-57, whether in solutions or in solids, is complicated by the possible existence of two tautomeric forms of the neutral species as illustrated in eq 1.



2a 2b (1)

The >NH and ≥N type nitrogens have quite different chemical shifts with the >NH type resonating at the higher field. Moreover, the >NH-type nitrogens of the neutral species usually resonate slightly *upfield* (~5–8 ppm) from their

position in the protonated imidazolium ion (species **3**). Thus,



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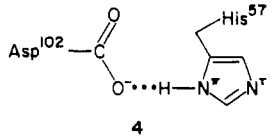
depending on the value of the equilibrium constant K (eq 1), ionization of the imidazole ring can result in an upfield, a downfield, or even no chemical shift change for either imidazole ring nitrogen.

Therefore, the charge state of the imidazole ring cannot be inferred from the chemical shift behavior of either ring nitrogen alone. However, studies in aqueous solutions have indicated that the average of the $^{15}N^{\pi}$ and $^{15}N^{\tau}$ chemical shifts is a reliable gauge of the protonation state of the imidazole ring (Bachovchin & Roberts, 1978; Roberts et al., 1982). In all derivatives examined, the average of the π- and τ-nitrogen chemical shifts was found to move 31 ppm from 202 ± 3 ppm to 171 ± 3 ppm as the imidazolium ion deprotonates. Thus, an average value of 171 ppm indicates an imidazole ring in the free-base form, whereas one of 202 ppm indicates the protonated imidazolium ion. Intermediate values indicate fractional protonation states.

In this study, we find that, in a lyophilized enzyme powder prepared from a solution with pH 5.0, an imidazole ring species with an average ^{15}N chemical shift value of 203 ppm predominates. As the "pH" is increased, this form gradually disappears and is replaced by a new species with an average ^{15}N chemical shift value of 175 ppm (Figure 3). On the basis of comparison with the behavior of the imidazole derivatives discussed above, it seems reasonable to attribute the low- and high-pH forms in the powder to a fully protonated and a fully neutral imidazole ring, respectively. Although we have not carried out a detailed pH titration, since the average chemical shift at pH 5.0 corresponds to that of a fully protonated imidazole ring and the average chemical shift at pH 9.0 corresponds to that of a neutral imidazole ring, we can state that the "pK_a" of this residue in lyophilized powders must be 7.0 ± 1.0. This corresponds to its pK_a value in the solution-state enzyme; therefore, the "pK_a" for His-57 in enzyme powders can be considered to be within the normal range for a histidyl residue. We turn next to the question of whether the triad structure remains intact in lyophilized powders.

The solution study of α-lytic protease (Bachovchin & Roberts, 1978) demonstrated that at high pH the N^{τ}–H tautomer of His-57 is strongly favored, as evidenced by the low-field chemical shift of N^{τ} (138 ppm) compared to N^{π} (204 ppm). Because this tautomer is not the preferred one for histidine (Blomberg et al., 1977; Reynolds et al., 1973), for almost all 4-substituted imidazole derivatives (Witanowski et al., 1972; Bachovchin & Roberts, 1978; Roberts et al., 1982), for histidyl residues in peptides (Reynolds et al., 1973; Wasylishen & Tomlinson, 1977), and for most histidyl residues in proteins (Wilber & Allerhand, 1977; Allerhand et al., 1977; Deslauriers et al., 1974), its predominance in α-lytic protease was attributed to a strong hydrogen-bond interaction with the nearby carboxylate anion of Asp-102 as indicated in structure **4**. Additional stabilization of this tautomer is also possible



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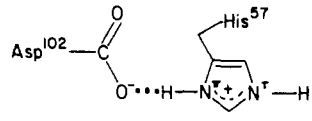
from hydrogen bonding between Ser-195 and N^{τ} of His-57 as indicated in structure **1**; however, whether this interaction contributes is uncertain at present (Matthews et al., 1977; Brayer et al., 1979). Nevertheless, that hydrogen bonding as depicted in **4** above is able to shift the tautomeric equilibrium in favor of the N^{π}–H tautomer has been confirmed recently by ^{15}N NMR studies of several imidazole model systems that form intramolecular hydrogen bonds (Roberts et al., 1982). The existence of the "unusual" N^{π}–H tautomer for histidyl residues in proteins seems to be emerging as a reliable indicator of hydrogen bonding to the imidazole ring (Walters & Allerhand, 1980).

A second unusual feature about the ^{15}N chemical shift behavior of His-57 noted in the solution study is that, for the protonated imidazolium ion of His-57, the chemical shift difference between N^{π} (191.6 ppm) and N^{τ} (204.2 ppm) is quite large (12.6 ppm) due to the abnormally low-field chemical shift of N^{π}. For cations of 4-substituted imidazole derivatives, the chemical shifts of both N^{π} and N^{τ} are normally near 200 ppm and do not differ from each other by more than 3.5 ppm. The low-field position of N^{π} and the resulting electrical asymmetry in the imidazole ring was assigned to hydrogen bonding between the carboxylate anion of Asp-102 and the protonated imidazolium ion of His-57 as depicted in structure **5**. The correctness of this assignment, based ori-



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ginally on analogy with the behavior of imidazole model systems known to be intermolecularly hydrogen bonded, has recently been confirmed by the very similar behavior observed in imidazole derivatives containing intramolecular hydrogen bonds (Roberts et al., 1982). Thus, for solutions the ^{15}N chemical shifts provide direct evidence for the existance of a hydrogen bond between the carboxylate of Asp-102 and His-57 at both high pH and low pH. In this work, we have demonstrated that the ^{15}N chemical shift behavior of His-57 in lyophilized enzyme powders corresponds quite closely to that previously observed in solution (Figure 3). The simplest explanation for this result is that Asp-102 and His-57 remain hydrogen bonded in the powders and that the strength of their interaction, whether at high pH or at low pH, is largely unaffected by lyophilization. This implies that the enzyme structure is very largely maintained even in an environment very different from aqueous solutions.

These results offer no support for the pK_a reversal or the modified charge-relay hypothesis (Hunkapillar et al., 1973) once advanced and widely accepted as the basis for the catalytic efficiency of these enzymes. The idea behind this proposal was that charge separation in the hydrophobic environment of the active site would be unfavorable, whether in the ground state or resting enzyme or in the transition state. Thus, according to this theory, addition of a proton to the Asp-His diad, whether on lowering the pH of the solution or on entering the transition state, should result in a neutral Asp/neutral His pair rather than a negatively charged Asp/positively charged His pair. This protonation scheme has been shown to be incorrect for resting enzymes in solution (Bachovchin & Roberts, 1978; Markley & Ibãnez, 1978; Markley et al., 1980). In this study we have demonstrated that it is also incorrect for lyophilized enzyme powders in which the active site environment might be expected to be considerably more hydrophobic than for aqueous enzyme solutions and thus more likely to exhibit this unusual pK_a reversal behavior.

In summary, we have examined lyophilized powders of α-lytic protease specifically ^{15}N enriched at His-57 by using solid-state NMR techniques. The "pH"-dependent behavior of the isotropic shifts of the imidazole ring nitrogens in these powders corresponds quite closely to the chemical shift behavior observed previously in solutions. Due to the exquisite sensitivity of the ^{15}N NMR signals to hydrogen bonding and tautomeric structure and to the fact that the behavior of the ^{15}N NMR signals for His-57 is quite unusual for histidyl and imidazole derivatives, yet very consistent with expectations for the hydrogen-bonded catalytic-triad structure of Asp-His-Ser, we conclude that this structure is maintained in detail in these lyophilized powders. Because these residues are brought together by the correct folding of the polypeptide chain, it seems likely that the solution and solid structures of α-lytic protease should be at least very similar. The ^{15}N NMR isotropic shifts further indicate that His-57 within the intact triad in lyophilized enzyme powders has what can be taken as a normal "pK_a" for a histidyl residue, undergoing a transition from protonated to neutral with a midpoint between pH 6.0 and pH 8.0.

Registry No. α-Lytic protease, 37288-76-9; L-histidine, 71-00-1; nitrogen-15, 14390-96-6.

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