Determination of a Low pK for Histidine-159 in the S-Methylthio Derivative of Papain by Proton Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: Proton NMR spectroscopy was used to study the ionization behavior of His-159 in a derivative of papain (papain-S-SCH_3). In this catalytically inactive derivative of papain, the active-site thiol group of Cys-25 is S-methylthiolated so that it cannot form a thiolate anion. The pH dependence of the chemical shift of the C^{ϵ}_1 H resonance of His-159 indicated a pK of 3.45 ± 0.07 at 45 °C in ^2H_2O with no added ions other than those required for titration. In acetate buffers at an ionic strength of 0.05, the pK increased to 3.87 ± 0.12. Conversion of papain-S-SCH_3 to active papain at pH* 4.17 (at 45 °C and an ionic strength of 0.05) caused the position of the C^{ϵ}_1 H resonance to change from a position indicative of partial protonation of His-159 to a position indicative of full protonation, consistent with the existence of an imidazolium–thiolate ion-pair interaction between His-159 and Cys-25 in the active enzyme.

Results of potentiometric difference titrations from this laboratory (Lewis et al., 1976) have established that ionization of the active-site thiol group in papain is linked to the ionization of a second group and that when ionization of the active-site thiol is blocked by methylthiolation, the pK of the second group is about 4. The second group was presumed to be His-159 on the basis of its proximity to Cys-25. This assignment is supported by the determination of a similar pK value from the pH dependence of fluorescence of papain-S-SCH_3[1] (Lewis et al., 1976). Although strong evidence has been presented that suggests that the observed pH-dependent fluorescence is due to quenching of the fluorescence of Trp-177 by the protonated form of the neighboring His-159 (Lowe & Whitworth, 1974), the possibility that the pH dependence of fluorescence reflects protonation of Asp-158 rather than His-159 has recently been raised (Zannis & Kirsch, 1978). The intrinsic uncertainty in the assignment of the ionization reflected in the fluorescence titrations made it important to determine the ionization behavior of His-159 in papain more directly by measuring the pH dependence of a property of His-159 itself. In this work proton NMR titrations are reported which indicate that His-159 in papain-S-SCH_3 does indeed have a low pK.

Experimental Procedures

Materials

Papain was isolated from crude dried papaya latex and purified by affinity chromatography by the method of Burke et al. (1974). The pure protein was stored as the mercuric derivative which was regenerated just prior to use by the addition of 11 mM 2-mercaptoethanol and 20 mM EDTA. The activated papain was subjected to a repetition of the affinity chromatography step used in the purification procedure in order to separate it from the other components in the activation mixture.

Papain-S-SCH_3 was prepared by a modification (Lewis et al., 1976) of the method of Smith et al. (1975). The protein was concentrated to 20 mg/mL by ultrafiltration on a Millipore PTGC membrane and was precipitated by addition of 10% (v/v) saturated KCl ("Suprapur", Merck), cooled for 1 h at 4 °C, and centrifuged at 20000g for 10 min.

1,3-Dibromoacetone-modified papain (DBA-papain) was prepared by a modification of the procedure of Husain & Lowe (1968). Freshly prepared active papain (124 mg, 5.3 μmol) in 90 mL of pH 5.6 0.05 M sodium acetate was reacted at room temperature with 1.14 mg (5.3 μmol) of 1,3-dibromoacetone in 1.5 mL of acetone. Since 5% of the catalytic activity toward N-α-benzoyl-L-arginine ethyl ester remained after 10 min, the reaction mixture was treated with an additional 0.114 mg (0.53 μmol of the reagent in 0.15 mL of acetone and was stirred for another 10 min, after which time the catalytic activity of the protein was undetectable. The protein was separated from other components in the reaction mixture by ultrafiltration (Five cycles of 2-fold dilutions with distilled water). Acid hydrolysis (6 N HCl, 110 °C, 20 h) followed by amino acid analysis showed 1 mol of histidine/mol of papain, based on 12 arginyl residues per molecule of papain, whereas active papain which had not been treated with DBA showed 2 mol of histidine/mol of papain.

Deuterium oxide (99.8%), deuterium chloride (37% in 99% ^2H_2O), and sodium deuteroxide (40% in 99% ^2H_2O) were obtained from Bio-Rad. The sources of other materials used in this work are described elsewhere (Lewis et al., 1976).

Methods

Concentrations of papain and derivatives of papain were determined from the absorbance at 280 nm with a molar absorptivity of 5.77×10^4 M^{-1} cm^{-1} (Skalski et al., 1973). The catalytic activity of papain was routinely assayed by one of two methods. The first method was performed by using N-α-benzoyl-L-arginine ethyl ester as described elsewhere (Lewis et al., 1976). In the second method the catalytic activity of papain was assayed with 100 μL of 10.2 mM N-α-benzoyl-L-arginine p-nitroanilide in 3 mL of pH 6.5 0.05 M phosphate buffer. After thermal equilibration at 25 °C of the substrate solution in the thermostated cell compartment of a GCA/

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[1] Abbreviations used: papain-S-SCH_3, the S-methylthio derivative of Cys-25 of papain; DBA, 1,3-dibromoacetone; DBA-papain, the 1,3-dibromoacetone-modified derivative of papain wherein the Cys-25 sulfur and His-159 N^{δ}_1 are cross-linked by dialkylation; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; TFE, poly(tetrafluoroethylene); FT, Fourier transformation; WEFT, water-eliminated Fourier-transform NMR; pH*, the glass electrode reading of pH in deuterium oxide without correction for isotope effects; FID, free induction decay; ppm, parts per million; $\Gamma/2$, ionic strength based on molar concentrations.

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McPherson EU 700 series spectrophotometer, 100 μL of the protein solution was added and the rate of change of absorbance was monitored at 410 nm. In this system the specific activity of papain was 0.16 unit/mg (1 unit = 1 μmol of *p*-nitroaniline/min), using the value $\Delta\epsilon_{410}$ = 8600 M^{-1} cm^{-1} for *p*-nitroaniline production.

^2H_2O Solutions of Proteins. A ^2H_2O solution of DBA-papain was prepared by repeated ultrafiltration and redilution of the protein solution in H_2O with a 2-fold quantity of 99.8% ^2H_2O. At least 13 cycles were used to bring the ^2H_2O level above 99.5%. A ^2H_2O solution of papain-*S*-SCH$_3$ was prepared by redissolving KCl-precipitated protein in 99.8% ^2H_2O and subjecting the resulting solution to repeated ultrafiltration and redilution with one volume of 99.8% ^2H_2O. Seven cycles were necessary to achieve a level of more than 99.5% ^2H_2O. Upon reaching the desired extent of enrichment, as measured by integration of the *HO*^{2}H peak in the NMR spectrum (using a Varian T-60 spectrometer) of the filtrate, solutions were concentrated to 2.5–3.0% protein by ultrafiltration and were filtered through 0.45-μm Millipore membranes.

pH measurements were made by using a Radiometer Model TTT1c pH meter equipped with a PHA 630T scale expander, and a combination electrode which was standardized at the appropriate temperature with 1:1 phosphate, and either biphthalate or borate, National Bureau of Standards primary standard solutions (Bates, 1964).

The pH* values of proton NMR samples (800 μL in small glass vials containing magnetic stirring bars) were measured after thermal equilibration of the sample in an oil bath consisting of a jacketted beaker through which water from a constant temperature bath was circulated. Sample temperatures were monitored with a Bailey BAT-8 digital thermometer and were controlled to within 0.4 °C of the temperatures at which proton NMR spectra were taken. When necessary, the pH* of samples was adjusted by slow addition of titrant (0.1–1.0 M ^{2}HCl or 0.01 M NaO^{2}H) to the stirred sample. When the titrant concentration was greater than 0.01 M, it was added at room temperature or below in order to avoid denaturation of the protein. Titrant was added from a Micro-Metric SB2 syringe microburet via TFE tubing which had been sealed at its end and pierced with a pin near the sealed end. When present, insoluble material was removed by centrifugation for 1 min on an Eppendorf Model 3200 desk-top centrifuge. The protein was then thermally reequilibrated, and its pH* again was read and recorded before being transferred to a clean, dry glass NMR tube (Wilmad Glass Co.). At intervals varying from 10 min to 1 h, the proton NMR sample was removed from the tube with a drawn-out glass pipet, and the pH* was checked, and, if necessary, was readjusted with titrant.

Proton NMR Spectroscopy. Spectra (100 MHz) at 35 and 45 °C were taken on a JEOL PFT-100 spectrometer interfaced to a JEOL EC-100 data system with a Texas Instruments 980B computer. The probe temperature was kept to within ±1 ° C with a thermocoupled temperature controller. The electromagnetic field was internally locked on the ^2H_2O signal of the solvent at 15 MHz; the sample was pulsed at 58 db with an irradiating frequency of about 100 MHz. Water-eliminated Fourier transform (WEFT: Patt and Sykes, 1972) was used to suppress the solvent *HO*^{2}H signal by employing a 180°–t_1–90°–t_2 pulse sequence, where t_2 = 1.35 s. The t_1 was set empirically to give a reduced solvent signal and usually fell between 1.20 and 1.30 s. Either 2048 or 4096 data points were used to accumulate the free induction decays for acquisition times of 1.35 s. Fourier transforms were carried out on the free induction decays; an exponential apodization constant, *n*, was used, where $-7 \leq n \leq 0$ and $FID_{calcd} = FID_{obsd} \exp(tn/T)$, where T = acquisition time and t = abscissa of the time-domain free induction decay. A block accumulation technique was used whereby every block of 100 time-domain transients was Fourier transformed and the frequency-domain spectrum obtained was added coherently to the previous sets based on overylay to the ^2H_2O peak of the solvent. Block accumulation compensates for long-term frequency drift. For each point in the titration curves, a total of 500–8000 transients were collected. The 150-MHz NMR spectra were collected on the Nicolet NT-150 spectrometer at the Purdue University Biochemical Magnetic Resonance Laboratory. The electromagnetic field was internally locked on the ^2H_2O signal of the solvent. Samples were irradiated every 5 s with single pulses (45° magnetization tip) until a total of 100–500 transients were collected and Fourier transformed for each 150-MHz NMR spectrum. The chemical shifts of peaks in the 150-MHz NMR spectra are expressed in parts per million (ppm) from an internal standard of 0.3–3 mM DSS. Over the entire pH range it was found that almost all peaks in the spectra of papain derivatives were independent of pH*. One of these peaks, which appeared in the aromatic region at 6.76 ppm downfield from internal DSS, was used as a reference to assign chemcial shifts for the peaks in the aromatic region of the 100-MHz spectra, which were taken in the absence of an internal shift standard.

Experimental data were fitted to equations by using a nonlinear least-squares program BMDX85, version of June 1972, written by Paul Sampson of the Health Sciences Computing Facility, The University of California, Los Angeles (Jennrich & Sampson, 1968), as adapted by the Statistics Research Laboratory, The University of Michigan. This program fits a specified function to data by means of stepwise Gauss–Newton iterations on the parameters.

Results

In papain-*S*-SCH$_3$ several peaks downfield of the aromatic envelope are seen in the region where histidyl C^{ϵ}_1 H resonances are expected (Figure 1). However, only two of these peaks, H1 and X2, exhibit pH-dependent chemical shifts in the pH range studied. As illustrated in Figure 2 the pH dependence of the H1 peak is much more pronounced than that of the X2 peak.

The instability of the protein at low pH values made it unfeasible to determine more than about 50–60% of the titration curves depicted in Figure 2, since NMR spectra could not be obtained below pH values of about 3.3 without irreversibly denaturing the protein. Because of the possibility of acid denaturation of protein during measurements of NMR spectra, control experiments were performed in order to verify that all reported pH dependencies of NMR spectra reflected reversible acid–base equilibria and not irreversible denaturation of the protein. Samples at the lowest pH values of the titrations, when readjusted to pH 4–5, gave NMR spectra indistinguishable from that of fresh samples of protein which had not been incubated at low pH values. Futhermore, samples used for obtaining NMR spectra at the lowest pH values (as well as all other pH values) exhibited after activation with DTT at least 75% of the specific catalytic activity of the native enzyme.

The pH dependence (Figure 2) of the chemical shift (δ_{obsd}) for the H1 peak was fitted to eq 1 to yield the chemical shift

$$\delta_{obsd} = \frac{\delta_{H^0} + 10^{(pK-pH^*)}\delta_{H^+}}{1 + 10^{(pK-pH^*)}} \quad (1)$$

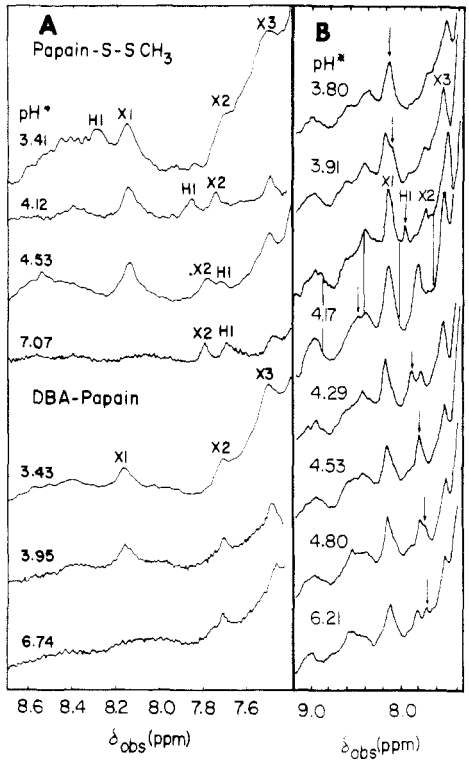

FIGURE 1: (A) Histidine region of represenatative 100-MHz WEFT proton NMR spectra of 2.5–3.0% papain-*S*-SCH_3 and DBA-papain in ^2H_2O at 45 °C and three pH* values. Chemical shifts were determined from a pH-independent peak at $\delta = 6.76$. X1, X2, and X3 are unassigned resonances. H1 is ascribed to the C^{ϵ}_1 H of His-159. (B) Histidine region of representative 150-MHz proton NMR spectra of papain-*S*-SCH_3 at several pH* values. Each spectrum is the sum of 500 transients at 45 °C in 0.05 M acetate buffered ^2H_2O. The arrow in each spectrum denotes the position of peak H1, the C^{ϵ}_1 H resonance of His-159. The two spectra connected by vertical lines (pH* 4.17) are before (upper spectrum) and after (lower spectrum) conversion of papain-*S*-SCH_3 to active papain by treatment with excess DTT.

Table I: Parameters Obtained from Titrations of Peak H1 in Papain-*S*-SCH_3[a]

parameter	35 °C[b] (Γ/2 < 0.01)	45 °C[b] (Γ/2 < 0.01)	45 °C[c] (Γ/2 = 0.05)
δ_{H^0} (ppm)	7.71 ± 0.01	7.68 ± 0.01	7.67 ± 0.02
δ_{H^+} (ppm)	8.85 ± 0.09	8.76 ± 0.09	8.51 ± 0.10
Δ (ppm)	1.14 ± 0.09	1.08 ± 0.09	0.84 ± 0.10
p*K*	3.39 ± 0.06	3.45 ± 0.07	3.87 ± 0.12

[a] Estimates of the parameters are given plus or minus standard deviations. [b] Determined by 100-MHz WEFT NMR. [c] Determined by 150-MHz NMR in 0.05 M acetate buffer.

of the protonated (δ_{H^+}) and unprotonated group (δ_{H^0}) and the apparent p*K* which describes the transition from the protonated to the unprotonated form (Table I). The fit of the pH dependence to eq 1 is consistent with the dependence of the chemical shift of the H1 peak on a single ionization. However, the possibility that the ionization reflected by the pH dependence of H1 is perturbed by other ionizations cannot be excluded, since only partial titration curves were obtained. Consequently, our interpretation that a single group is responsible for the pH dependence of the H1 peak may be oversimplified. Nevertheless, the p*K* values reported in Table I describe at least to a first approximation the ionization behavior of a single group in papain-*S*-SCH_3. The limited data listed in Table I suggest that the p*K* of this group increases with ionic strength but remains nearly constant upon increasing the temperature.[2]

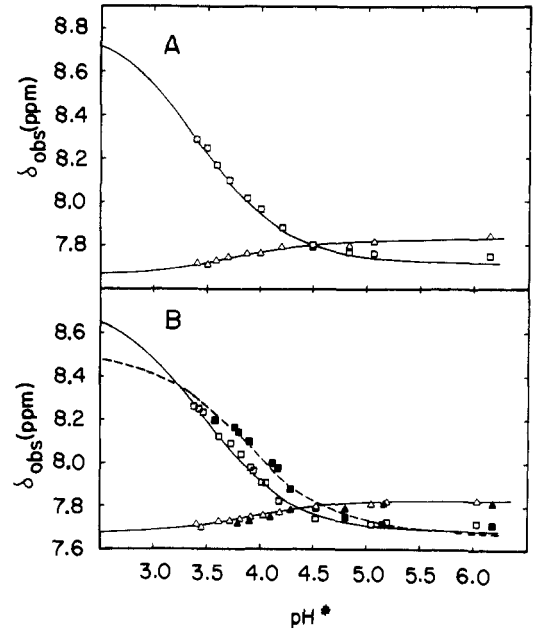

FIGURE 2: pH dependencies of the chemical shifts of peaks H1 (squares) and X2 (triangles) in the proton NMR spectra of 1–1.3 mM papain-*S*-SCH_3. (A) Spectra collected at 35 °C at 100 MHz in ^2H_2O. (B) Spectra collected at 45 °C either at 100 MHz in ^2H_2O (open symbols) or at 150 MHz in 0.05 M acetate buffered ^2H_2O (closed symbols). The nonlinear least-squares fits of eq 1 to data from the 100- and 150-MHz NMR spectra are shown by the solid and dashed lines, respectively. Values for the fitted parameters of eq 1 for the H1 peak are listed in Table I. Peak H1 is ascribed to the C^{ϵ}_1 H resonance of His-159.

The resonance represented by peak H1 is assigned to the C^{ϵ}_1 H of His-159 in papain-*S*-SCH_3. This assignment is based upon three criteria: (a) the direction and span of the chemical shift change between low and high pH, (b) the decrease of the H1 signal due to exchange with deuterium from the solvent, and (c) the behavior of the peak in another derivative of papain in which His-159 is modified.

(a) The change in chemical shift of 0.8–1.1 ppm obtained from fitting the pH dependence of H1 to eq 1 is of the same magnitude and direction as the 1.0 ± 0.2 ppm downfield movement in chemical shifts usually seen in the C^{ϵ}_1 H resonance on protonation of histidyl residues in proteins (Markley, 1975). Peak X2, on the other hand, shifts upfield when the pH is decreased so that its pH dependence cannot reasonably be ascribed to the C^{ϵ}_1 H of a histidyl residue. Ionizations of neighboring groups in proteins have been shown to cause changes in chemical shifts for C^{ϵ}_1 H resonances (Cohen and Shindo, 1975; Markley and Ibañez, 1978), and one could argue that the pH dependence of H1 reflects such an ionization. There is no known precedent, however, for a neighboring ionization causing a 0.8–1.1 ppm chemical shift change in the C^{ϵ}_1 H resonance for a histidyl residue. Studies by Shrager et al. (1972) indicate a perturbation of less than 10% of the total chemical shift change of the C^{ϵ}_1 H resonance by neighboring ionizations in histidine and other low molecular weight derivatives of this amino acid. Naturally, we cannot exclude the possibility that another ionization in papain-*S*-SCH_3 causes

[2] Proton NMR is usable only within a temperature range of 10 °C or so with papain and its derivatives. At high temperatures (>45 °C), the highly concentrated protein undergoes heat denaturation at the low pH (pH <4) needed to find the p*K*, whereas at lower temperatures (<35 °C), spectral line broadening adversely affects the resolution of NMR signals. In light of the limited temperature range and the experimental error associated with the NMR titrations, determination of a precise heat of ionization from the temperature dependence of its p*K* is impractical.

a 1-ppm chemical shift change in the C^{ϵ}_1 H resonance of the protonated form of His-159. For example, ionization of a group in the enzyme might mediate a conformational change which results in an altered environment of the C^{ϵ}_1 H and causes it to exhibit a chemical shift change corresponding in direction and magnitude to that expected for deprotonation of an imidazolium side chain. If such a coincidence occurred, however, the C^{ϵ}_1 H resonance should exhibit another change in chemical shift at higher pH values where His-159 might be expected to undergo deprotonation. No such change in the chemical shift was seen, however, for the H1 peak in the pH range 6–8. The decreased solubility of papain-*S*-SCH$_3$ and the decreased resolution of the spectra near the isoelectric point of papain-*S*-SCH$_3$ precluded obtaining useful NMr spectra above pH* 8.

(b) Slow chemical exchange of the C^{ϵ}_1 H for deuterium is a characteristic of histidyl residues in proteins [Markley (1975) and references cited therein]. Incubation of papain-*S*-SCH$_3$ in ^2H_2O at pH* 8.5 for 11 days at 45 °C resulted in loss of more than half of the area of peak H1, whereas peaks X1, X2, and X3 maintained essentially the same areas. In another experiment a similar rate of loss of the H1 peak was also observed when papain-*S*-SCH$_3$ was incubated at pH* 4.75.[3] The decrease in the integral of peak H1 was used to distinguish it from peak X2 in the pH range where chemical shifts of the two resonances approach each other and exchange relative positions in the NMR spectrum.

(c) Husain & Lowe (1968) have shown that in papain the sulfur atom of Cys-25 and the N^{δ}_1 atom of His-159 can be cross-linked by dialkylation with 1,3-dibromoacetone. Comparison of NMR spectra (Figure 1) of this derivative (DBA-papain) with that of papain-*S*-SCH$_3$ at three pH values indicates that peak H1 is absent from the spectrum of DBA-papain at all pH values. The missing resonance may have been broadened or shifted to another spectral region so that it is no longer detectable. Shindo et al. (1976) observed an analogous loss of the resonance upon carboxymethylation of His-119 in ribonuclease S and pointed out that restriction of motion of the histidyl side chain may be responsible for the loss of the C^{ϵ}_1 H resonance. It is unlikely that cross-linking of Cys-25 and His-159 introduced rigidity throughout the molecule in regions remote from the active site. Such an effect would be expected to result in extensive line broadening of many peaks, which was not observed. Thus, it is most sensible to conclude that the chemical environment immediately adjacent to the atom responsible for the H1 resonance is altered in DBA-papain, namely C^{ϵ}_1 H of His-159. Interestingly, the chemical shift of X2 becomes independent of Ph in DBA-papain. The alteration in the titration behavior of X2 in DBA-papain indicates that the atom responsible for this resonance also is near the active-site region. Peaks X1 and X3 appear the same in spectra of DBA-papain and papain-*S*-SCH$_3$. When the pH* of solutions of papain-*S*-SCH$_3$ is increased above pH* 6.5, peak X1 moves slightly upfield with pronounced line broadening. Although there is insufficient evidence at this time to assign peak X1, it may well represent the C^{ϵ}_1 H resonance of His-81, since it exhibits the line-broadening characteristic of a buried histidyl residue in which protonation and deprotonation is slow.

Removal of the methylthio group from papain-*S*-SCH$_3$ by reaction with dithiothreitol appeared to cause the H1 peak to disappear from 100-MHz NMr spectra. In later experiments, however, wherein better resolved 150-MHz spectra of papain-*S*-SCH$_3$ and papain in 0.05 M acetate buffers were compared at the same pH value, it could be ascertained that the H1 signal did not disappear, but instead underwent a shift in its resonance frequency. The shift in the H1 peak at pH* 4.17 from 7.97 ppm in papain-*S*-SCH$_3$ to 8.47 ppm in papain is depicted in Figure 1B. The sensitivity of the chemical shift of the H1 signal to substitution of the thiol group of Cys-25 is further evidence for its being the C^{ϵ}_1 H resonance of the neighboring His-159.

The chemical shift of peak H1 in active papain is close to the chemical shift (δ_{H^+} = 8.51) estimated for the C^{ϵ}_1 H resonance of the protonated form of His-159 in papain-*S*-SCH$_3$ in 0.05 M acetate buffer (Table I). This observation suggests that His-159 in active papain is fully protonated at pH* 4.17 whereas His-159 in papain-*S*-SCH$_3$ is only about 33% protonated at pH* 4.17. Collecting spectra of active papain above pH* 8 where His-159 would be expected to titrate (and cause the C^{ϵ}_1 H resonance to shift upfield) was not possible due to the low solubility of papain in the alkaline pH range.[4] The C^{δ}_2 H resonance of His-159 was not observed in our spectra. Resonances of the C^{δ}_2 H usually appear about 1 ppm upfield of C^{ϵ}_1 H resonance lines [see examples in Markley (1975)]. Such a chemical shift would cause the C^{δ}_2 H resonance of His-159 to fall under the large aromatic envelope of resonances where it would not be observable.

Discussion

The dependence of the chemical shift of the histidyl C^{ϵ}_1 H resonance in proton NMR spectra on the protonation state of the histidyl residue has been used to study the ionization behavior of histidyl residues in proteins [Markley (1975) and work cited therein]. The NMR titrations reported in this work indicate that the p*K* of His-159 is about 3.9 (Γ/2 0.05, 45 °C) when ionization of the thiol group is blocked. These titrations are the first measurements of the pH dependence of an intrinsic property of the active-site histidyl residue. They support previous assignments of a p*K* of about 4 for His-159 in thiol-blocked derivatives of papain (e.g., Lewis et al., 1976; Bendall & Lowe, 1976a,b; Allen & Lowe, 1973; Nicholson & Shafer, 1980) which were based on less direct evidence, namely, the proximity of His-159 to the active site and the observation that spectral properties of Trp-177 and reporter groups linked to the active-site thiol were affected by a p*K*-4 ionization.

It should be noted that His-159 probably does not have a low p*K* in the native enzyme wherein ionization of Cys-25 is not blocked. Previous observations suggest the existence of an ion-pair interaction between Cys-25 and His-159 in the native enzyme (Jolley & Yankeelov, 1972; Polgar, 1973; Lewis et al., 1976, 1978; Bendall & Lowe, 1976a,b; Sluyterman & Wijdenes, 1976; Nicholson & Shafer, 1980; Polgar, 1977, and work cited therein). Potentiometric difference titrations (Lewis et al., 1976) and fluorescence titrations of papain- and thiol-blocked derivatives of papain (Sluyterman & Wijdenes, 1976) suggest that in the unmodified native enzyme Cys-25

[3] The exchangeability of the C^{ϵ}_1 H for deuterium at pH 4.75 is consistent with His-159 having a low p*K* and being predominantly unprotonated at pH 4.75, since under the conditions used the rate of exchange of the C^{ϵ}_1 H is proportional to the fraction of histidine present in the unprotonated form. The exchange of the C^{ϵ}_1 H via attack on the unprotonated and protonated forms of histidine by hydroxide ion and water, respectively, is negligible (see Matsuo et al., 1972).

[4] Preparation of succinyl-papain, a soluble derivative of papain that is catalytically active, has allowed further study of the pH dependence of the C^{ϵ}_1 H resonance of His-159 at pH* values above 8.0 (see the following paper).

has a pK of about 3-4 and His-159 has a pK of about 8.5, whereas the pK of His-159 is about 4 in derivatives of papain wherein the negative charge on the sulfur atom of Cys-25 is neutralized by alkylation, alkylthiolation, or acylation. Consistent with these pK assignments is the observation that the chemical shift of the C^{ϵ_1} H resonance of His-159 indicates full protonation of this residue in active papain at pH* 4.17 and only partial protonation of His-159 in papain-S-SCH_3 at this pH* value.

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Effect of Cysteine-25 on the Ionization of Histidine-159 in Papain As Determined by Proton Nuclear Magnetic Resonance Spectroscopy. Evidence for a His-159–Cys-25 Ion Pair and Its Possible Role in Catalysis†

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ABSTRACT: Papain was succinylated in order to increase its solubility above pH 8 so that proton NMR spectroscopy could be used to study the ionization of His-159 at the active site of the enzyme. The pH dependence of NMR spectra of catalytically active succinyl-papain and the methylthio derivative of the active-site cysteinyl residue of succinyl-papain (succinyl-papain-S-SCH_3) were determined between pH 6 and 10. The pH dependence of the C^{ϵ_1} H resonance of His-159 in catalytically active succinyl-papain indicates that His-159 has a pK of about 8.6 in the catalytically active form of the enzyme. The position of this resonance in succinyl-papain-S-SCH_3 indicates that when the active-site cysteinyl residue is methylthiolated, His-159 is completely deprotonated between pH 6 and 10. This result is taken as evidence for an imidazolium–thiolate ion-pair interaction between His-159 and Cys-25 wherein neutralization of the charge on the thiolate anion by methylthiolation would be expected to cause a marked decrease in the pK of His-159. A possible catalytic role for the ion pair in the acylation step in papain-catalyzed reactions is proposed wherein attack of a substrate by the imidazolium–thiolate ion pair is accompanied by an increase in the acidity of the imidazolium group that facilitates expulsion of the leaving group of the substrate.

In an earlier attempt to characterize the ionization behavior of the thiol group at the active site of papain, we determined titrimetrically the pH dependence of the difference in proton content of papain and papain-S-SCH_3[1] (Lewis et al., 1976). These studies clearly showed that ionization of the active-site thiol group was linked to the ionization of another group which

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[1] Abbreviations used: papain-S-SCH_3, the S-methylthio derivative of Cys-25 of papain; succinyl-papain-S-SCH_3, the S-methylthio derivative of Cys-25 of succinyl-papain; pH*, the glass electrode reading of pH in deuterium oxide without correction for isotope effects.

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