Perturbations in the Free Energy and Enthalpy of Ionization of Histidine-159 at the Active Site of Papain As Determined by Fluorescence Spectroscopy†

Frederick Allen Johnson, Sidney D. Lewis, and Jules A. Shafer*

ABSTRACT: Fluorometric titrations of papain, succinyl-papain, and the corresponding methylthio derivatives of Cys-25 (papain-*S*-SCH_3 and succinyl-papain-*S*-SCH_3) were determined. Removal of the methylthio group from Cys-25 resulted in an increase of approximately 4 p*K* units in the fluorometrically determined p*K* value. The correspondence between the ionization behavior as determined by proton NMR and fluorometric titrations indicated that fluorescence titrations reflect the ionization behavior of His-159 in both the active enzyme and the methylthio derivative. The ionic strength dependence of the p*K* was analyzed in terms of simple electrostatic theory and was shown to be consistent with the charge on the protein. The temperature dependence of the p*K* values of His-159 indicated an increase in the heat of ionization from about 0 to 8 kcal/mol upon removal of the methylthio blocking group from Cys-25. Measurements of the effect of solvent on the p*K*'s and heats of ionization of simple model compounds indicated that the observed shift in enthalpy of ionization of His-159 upon removal of the methylthio group from Cys-25 is not unreasonable in light of the accompanying perturbation of more than 4 p*K* units in the p*K* of His-159. The perturbations in enthalpies and free energies are attributed to formation of an ion pair. The ionization behavior of His-159 in thiol-blocked derivatives of papain is consistent with the involvement of His-159 in the deacylation step in papain catalysis.

The pH dependence of the catalytic activity of the plant protease papain reflects the influence of three ionizable groups (Lewis et al., 1978; Sluyterman & Wijdenes, 1973; Whitaker & Bender, 1965). The triad contains the active-center thiol group of Cys-25, the imidazolyl group of His-159, and probably the carboxyl group of Asp-158. Potentiometric difference titrations and NMR titrations indicate that the predominant ionization pathway for Cys-25 and His-159 is one in which Cys-25 has an unusually low p*K* of about 3 to 4, and His-159 has a p*K* of about 8.5 (Lewis et al., 1976, 1981; Johnson et al., 1981; Sluyterman & Wijdenes, 1976). These studies, which indicate that His-159 and Cys-25 exist predominantly as an imidazolium–thiolate ion pair at physiological pH values, support earlier kinetic studies which suggested the existence of such an ion-pair interaction (Jolley & Yankeelov, 1972; Polgar, 1973).

NMR and fluorescence titrations of derivatives of papain indicate that blocking ionization of Cys-25 by alkylation, alkylthiolation, or alkylmetalation causes the p*K* of the adjacent His-159 to decrease from 8.5 to about 4 (Johnson et al., 1981; Lewis et al., 1976, 1981; Bendall & Lowe, 1976a,b; Sluyterman and Wijdenes, 1976; Lowe & Whitworth, 1974; Sluyterman & de Graaf, 1970). By analogy one would also expect a p*K* of about 4 for His-159 in the thiol ester acyl-enzyme intermediate which forms during catalysis. Consistent with this view is the observation that deacylation of acyl-papain intermediates depends on the basic form of an ionizable group with a p*K* of 3.9–4.6 (Williams et al., 1972; Hinkle & Kirsch, 1970, 1971; Lowe, 1970; Brubacher & Bender, 1966; Whitaker & Bender, 1965). However, the ionizable group that modulates the deacylation step in catalysis has been shown to have an enthalpy of ionization near zero, which is more characteristic of a carboxylic acid than of an imidazolyl group (Hinkle & Kirsch, 1971). On the basis of the low p*K* value, the near-zero heat of ionization, and the high Hammett ρ value for deacylation of substituted benzoyl-papain derivatives, Zannis & Kirsch (1978) have argued that the ionizable group that modulates deacylation of acyl-papain derivatives is a carboxyl group and not an imidazolyl group. Zannis & Kirsch (1978) have also questioned the conclusion of Lowe & Whitworth (1974) that the p*K* = 4 group seen in the fluorometric titrations of thiol-blocked derivatives of papain is His-159.[1]

In this paper we report comparisons of NMR and fluorometric titrations of papain and papain derivatives supporting the view that the pH dependence of fluoroscence of papain reflects ionization of His-159 in the active enzyme and other inactive derivatives wherein ionization of Cys-25 is blocked. We also report the use of fluorometric titrations to determine precisely the temperature dependence of the ionization of His-159. These studies indicate a near-zero heat of ionization for this residue in papain-*S*-SCH_3,[2] consistent with His-159 being the p*K* = 4 group modulating the deacylation step in catalysis.

Experimental Procedures

Materials

Imidazole (Aldrich) was recrystallized from benzene and sublimed in vacuo. 4(5)-Bromoimidazole was from Chemical Dynamics Corp. 2-Picoline and 2,6-lutidine were obtained from Aldrich and were redistilled prior to use. Sources of all other materials used in this work are reported elsewhere

† From the Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109. *Received May 21, 1980.* This study was made possible by Research Grant AM09276 and Training Grant GM00187 from the National Institutes of Health. Part of this work is described in a Ph.D. dissertation submitted by F.A.J. to the Graduate School of The University of Michigan, 1979.

[1] Lowe & Whitworth (1974) have presented evidence that the pH dependence of the fluorescence of papain reflects quenching of the fluorescence of Trp-177 by the protonated form of the adjacent His-159.

[2] Abbreviations used: papain-*S*-SCH_3, The *S*-methylthio derivative of Cys-25 of papain; succinyl-papain, the derivative of papain wherein the amino groups have been modified by succinic anhydride; DBA-papain, the 1,3-dibromoacetone-modified derivative of papain wherein Cys-25 sulfur and His-159 $N^{\epsilon 1}$ are cross-linked by dialkylation.

0006-2960/81/0420-0052$01.00/0

(Johnson et al., 1981; Lewis et al., 1981).

Methods

pH dependencies of fluorescence (excitation 280 nm, emission 350 nm) of papain (papain-*S*-SCH_3), and succinyl-papain-*S*-SCH_3 in buffered solutions at a protein concentration of 4 μM were determined by using a ratio fluorometer equipped with Schoeffel GM100 monochromators for excitation and emission. The instrument was built by Dr. David Ballou and Mr. Gordon Ford of this department. Constant temperature ± 0.2 °C was maintained by circulating water from a thermostated bath through the cell holder. When the temperature was below 20 °C, dry air was blown into the cell compartment to prevent fogging of the cell windows.

Buffers were made by using acetic acid–sodium acetate (pH 3–5.6), sodium monophosphate–potassium diphosphate (pH 5.9–8.0), Tris-HCl (pH 8.0–9.0), and sodium bicarbonate–sodium carbonate (pH 9.2–10). Tris and glycine buffers were used for titrations of succinyl-papain in ^2H_2O. KCl was used where necessary to ensure constant ionic strength. All buffers and protein solutions were filtered through 0.45-μm Millipore filters into clean, dry vials before use in fluorometric measurements.

Samples were prepared by pipetting 3 mL of the appropriate buffer into a clean, dry fluorescence cell. (Samples for near-zero ionic strength titrations in weakly buffered solutions were prepared by pipetting 3 mL of pure ^2H_2O or H_2O and 0–100 μL of 2 M acetic acid in ^2H_2O or H_2O, respectively, into a cell.) A 150-μL aliquot of 0.2% papain-*S*-SCH_3 in ^2H_2O or H_2O was pipetted into the cell and stirred. Upon thermal equilibration the fluorescence intensity of the protein solution was determined. The intensity of a tryptophan standard solution was then rechecked to ensure that the fluorometer output had not drifted. The pH of the protein solution was then measured. This procedure was repeated to obtain a set of fluorescence intensities between pH 3.1 and 10.

Although papain and derivatives of papain undergo time-dependent irreversible denaturation at low pH values, the pH dependencies reported here reflect reversible acid–base equilibria of undenatured protein. Back titration of samples at the lowest and highest pH values to pH 5.3 results in recovery of the expected fluorescence. Furthermore, samples at the lowest and highest pH values of the fluorescence titrations exhibit catalytic activities upon activation which are within 90% that of the fully active enzyme provided they are transferred to the neutral activating buffer immediately after measurement of fluorescence.

pH measurements for determination of p*K*'s and enthalpies of ionization were made in thermostated vessels in water and in 50% (w/v) water/methanol. Values of $-\log [H^+]$ in 50% (w/v) water/methanol were calculated from the pH readings (B) using eq 1 (Van Uitert & Haas, 1953) where $\log U_H$ is

$$-\log [H^+] = B + \log U_H \quad (1)$$

a correction factor obtained from the mean deviation of pH meter readings of HCl standard solutions (at the ionic strength of the sample) in the mixed organic solvent from the known $-\log [H^+]$ values, assuming complete dissociation of HCl in 50% (w/v) water/methanol at these concentrations of HCl (Shedlovsky & Kay, 1956). The value of $\log U_H$ was independent of the value of $[H^+]$ for the HCl standards used (0.003 M to 0.02 M HCl). Equation 2 was used to convert values

$$pa_H^* = -\log (m_{H^+} {}_s\gamma_H) \quad (2)$$

of $-\log [H^+]$ to a_H^*, the hydrogen ion activity referred to the standard state in the particular solvent under consideration. It was assumed that in dilute solution $[H^+] \simeq m_H$, the molality of hydrogen ion (Bates, 1964). The value of ${}_s\gamma_H$, the activity coefficient for the salt effect of hydrogen ion in the particular solvent under consideration, was determined from eq 3 (Bates, 1964), where å = 9 Å, the ion-size parameter for hydrogen ion, D is the dielectric constant of the solvent, d^0 is the density of the solvent, μ is the ionic strength, T is the absolute temperature (Kelvin), and $z_H = +1$, the charge of the hydrogen ion. Values of D for 50% methanol/water were calculated from eq 4 (Akerlof, 1932), where $\log a = 1.7512$, $b = -0.00234$, and T is the temperature in degrees Celsius. The value d^0 is from Wolf et al. (1971).[3] Values of $-\log {}_s\gamma_H$ were between 0.03 and 0.13 at the ionic strengths used (0.02–0.078). The temperature dependence of $-\log {}_s\gamma_H$, which was <0.01 per 30 °C, could be neglected. Values of pa_H in water were taken directly as the standardized pH meter readings. All other methods used in this work have been described previously (Johnson et al., 1981; Lewis et al., 1981).

$$-\log {}_s\gamma_H = \frac{(1.82455 \times 10^{-6}) z_H^2 \sqrt{\mu d^0}}{(DT)^{3/2}[1 + 50.2904(DT)^{-1/2} å \sqrt{\mu d^0}]} \quad (3)$$

$$\log D = \log a + b(T - 20) \quad (4)$$

Results

To establish more firmly the identity of the group responsible for the pH dependence of fluorescence, we have compared NMR titrations of His-159 with fluorescence titrations under similar conditions. Figure 1 depicts fluorescence titration curves for papain-*S*-SCH_3, active papain, succinyl-papain-*S*-SCH_3, and active succinyl-papain at an ionic strength of 0.05 in ^2H_2O at 45 °C. p*K* values were determined by fitting the pH dependence of the observed fluorescence (f_{obsd}) to eq 5,

$$f_{obsd} = \frac{f_{H^0} + 10^{(pK - pH^*)} f_{H^+}}{1 + 10^{(pK - pH^*)}} \quad (5)$$

where f_{H^0} and f_{H^+} are the fluorescence intensities at the high- and low-pH plateau, respectively. The p*K* value of 4.14 ± 0.05 obtained from the fit of eq 5 to the data for papain-*S*-SCH_3 (below pH 7) is in reasonable agreement with the value of 3.87 ± 0.12 for the p*K* of His-159 determined under similar conditions from an NMR titration (Johnson et al., 1981). An NMR titration carried out in the absence of added ions other than those required for pH adjustment indicates a lower p*K* of 3.45 ± 0.07 (Johnson et al., 1981) for His-159 in papain-*S*-SCH_3. This value is in reasonable agreement with a p*K* of 3.25 ± 0.04 observed for the fluoresence quenching group in the absence of added ions (Table I). As shown in Figure 1, papain-*S*-SCH_3 and succinyl-papain-*S*-SCH_3 exhibit a decrease in fluorescence above pH 7. Sluyterman & de Graaf (1970) have observed this effect with other thiol-blocked derivatives of papain and have attributed the quenching of fluorescence to ionization of tyrosyl side chains. Measured fluorescence intensities for active papain and succinyl-papain were corrected for quenching above pH 7 by using a correction factor estimated from the observed decrease in fluorescence of their methylthio derivatives as suggested by Sluyterman & de Graaf (1970). The corrected fluorescence titration curves (depicted in Figure 1) indicate that removal of the methylthio blocking group from Cys-25 causes the fluorometrically de-

[3] Since the volume of the buffers in water and 50% (w/v) water/methanol did not change detectably from 5 to 35 °C, the same value of the density of these solvents at 20 °C was used for calculations at both temperatures.

Table I: Fluorometrically Determined pK Values of Papain and Some Papain Derivatives

protein	T (°C)	ionic strengths					
		<0.01[a]	0.01	0.05	0.10	0.15	0.3
papain-S-SCH_3	5				4.16 ± 0.04		
	15			4.13 ± 0.05		4.32 ± 0.05	
	25	3.09 ± 0.05	3.70 ± 0.04		4.23 ± 0.05		4.48 ± 0.06
	25	3.10 ± 0.04[b]					4.47 ± 0.05[b]
	29			4.13 ± 0.05		4.32 ± 0.04	4.51 ± 0.06
	45	3.35 ± 0.04			4.20 ± 0.04		
	45	3.25 ± 0.04[b]		4.14 ± 0.05[b]			
succinyl-papain-S-SCH_3	25						4.84 ± 0.04
	45			4.85 ± 0.03[b]			4.93 ± 0.08
papain	15			8.75 ± 0.03			
	25						8.39 ± 0.03
	25						8.27 ± 0.04[b]
	29			8.47 ± 0.05			
	45			8.18 ± 0.02[b]			
succinyl-papain	25		8.52 ± 0.08				
	45			8.60 ± 0.03[b]			

[a] No added ions were present except those needed for titration. The effective average ionic strength is estimated as being roughly equal to the hydrogen ion concentration when pH = pK. [b] These pK values were determined in ^2H_2O and are not corrected for the difference in response of the glass electrode in ^2H_2O.

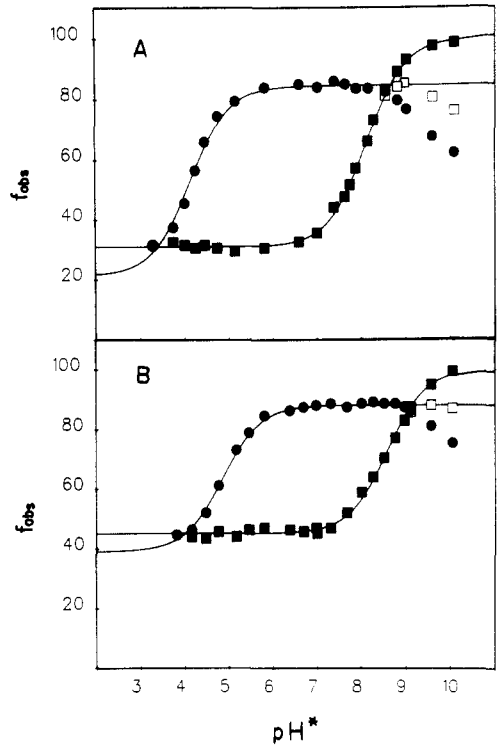

FIGURE 1: Fluorescence titrations in deuterium oxide at 45 °C and an ionic strength of 0.05. (A) Fluorescence titrations of papain-S-SCH_3 (circles) and active papain (squares). (B) Fluorescence titrations of succinyl-papain-S-SCH_3 (circles) and active succinyl-papain (squares). When necessary, fluorescence values were corrected for the quenching of fluorescence caused by ionization of tyrosyl residues. The corrected fluorescence values were obtained by dividing the observed fluorescence values (open squares) by f_s/f_p, where f_s is the fluorescence value for the methylthio derivative at the pH value of interst and f_p is the fluorescence for the methylthio derivative at the pH 6–8 plateau. The fit of the data to eq 5 yielded pK values for papain-S-SCH_3 (pK = 4.14 ± 0.05), active papain (pK = 8.18 ± 0.02), succinyl-papain-S-SCH_3 (pK = 4.85 ± 0.03), and active succinyl-papain (pK = 8.60 ± 0.03). The higher pK seen with succinyl-papain (relative to papain) is attributed to the greater negative charge density of succinyl-papain.

termined pK values to increase by about 4 pK units.

NMR titrations of papain-S-SCH_3 and active succinyl-papain indicate that upon removal of the methylthio group from Cys-25, His-159 exhibits the same striking increase in pK observed in the fluorescence titrations.[4] The fluorometrically determined pK of 8.60 ± 0.03 for succinyl-papain is in excellent agreement with the pK of 8.62 ± 0.06 determined for His-159 in succinyl-papain by an NMR titration (Lewis et al., 1981). The correspondence between the fluorometrically determined pK values and the ionization behavior of His-159 as determined by NMR titrations provides compelling evidence in support of the conclusion that the pH-dependent fluorescence observed in papain and derivatives of papain reflects ionization of His-159.

Results of fluorescence titrations performed at several temperatures and ionic strengths are listed in Table I. The observed increase in pK of His-159 in papain-S-SCH_3 with increasing ionic strength suggests that ionic interactions are at least partially responsible for its low pK. For analysis of the effect of ionic strength on fluorometrically determined pK values, the measured pK was defined by eq 6, where pK^0

$$pK = pK^0 + \log \gamma_A - \log \gamma_{AH} \quad (6)$$

represents the thermodynamic pK value of the group of interest and γ_A and γ_{AH} represent the activity coefficient of the protein when the group under study is unprotonated and protonated, respectively. Equation 6 was derived with the assumption that the measured pH values closely approximate values of $-\log a_H$ so that the fluorometrically determined pK values approximate values of $-\log (a_H[A]/[AH])$, where the square brackets indicate concentrations. If one assumes a spherically symmetrical charge distribution for the protein ion, one should be able to use eq 7–9 to predict the dependence on ionic

$$\log \gamma_A = \frac{(z-1)^2 e^2}{4.606 \bar{k} TD}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) \quad (7)$$

$$\log \gamma_{AH} = \frac{z^2 e^2}{4.606 \bar{k} TD}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) \quad (8)$$

strength (μ) of γ_A and γ_{AH} (Edsall & Wyman, 1958), where

$$\kappa = \left[\frac{8\pi N e^2}{1000 kDT}\right]^{1/2} \mu^{1/2} \quad (9)$$

z is the charge on the protein; e, the electronic charge; $\bar{k}$,

[4] The solubility of active papain in alkaline solutions and the solubility of succinyl-papain-S-SCH_3 in acid solution were insufficient to determine the ionization behavior of His-159 in these proteins by using NMR spectroscopy.

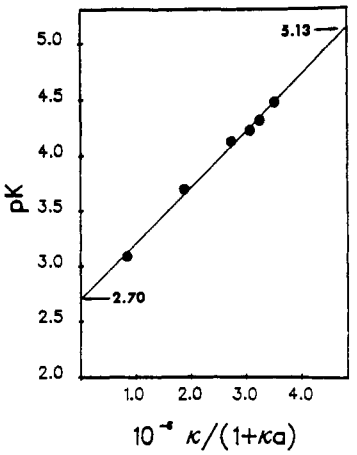

FIGURE 2: Dependence of the pK of His-159 in papain-S-SCH$_3$ on the ionic strength. The parameter κ was calculated from eq 9. Equation 10 was used to relate the slope of the plot in this figure to z, the average charge of the protein. The right vertical axis is at $\kappa/(1 + \kappa a) = 1/b$, so that its intersection with the plotted line yields a pK value for His-159 if the protein were uncharged. The intersection of the left vertical axis at $\kappa/(1 + \kappa a) = 0$ with the plotted line yields the pK of His-159 at an ionic strength of zero where none of the charge of the protein would be neutralized.

Boltzmann's constant; D, the dielectric constant; T, the absolute temperature; a, the sum of the ionic radius of the protein (b) and its counterion; and N, Avogadro's number. Substituting the expressions for log γ_A and log γ_{AH} in eq 6, one obtains eq 10. The value of the ionic radius of papain, b (20.8 Å), was estimated from eq 11, which assumes that the ionic

$$pK = pK^0 - \frac{(2z-1)e^2}{4.606\bar{k}DT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) \quad (10)$$

$$b = \left[\frac{3M_r}{4\pi N}(\bar{\nu}_2 + 0.25)\right]^{1/3} \quad (11)$$

radius of the protein is equivalent to that of a sphere of the same mass and density of papain with 25% hydration. Values of 23 406 and 0.723 cm^3/g were used for the molecular weight (M_r) and partial specific volume ($\bar{\nu}_2$) of papain (Mitchel et al., 1970). The value of the ionic radius of the counterions was taken as 2 Å, the ionic radius of chloride ion, to yield a value of 22.8 Å for a.

The linear dependence of the value of the measured pK on $\kappa/1 + \kappa a)$ predicted by eq 10 is illustrated in Figure 2. A value of 16.8 for z, the charge on protein ion AH, was calculated from the slope of the plot in Figure 2. This value of z is in reasonable agreement with values of about 16–21 estimated for z in the pH range corresponding to pK values plotted in Figure 2 (pH 3.7–4.5).[5] Extrapolation of the line in Figure 2 to a point where $\kappa/(1 + \kappa a)$ is numerically equal to $1/b$ and pK = pK^0 suggests that if the charge on the protein were zero, the measured pK would be 5.13. Interestingly, succinyl-papain-S-SCH$_3$, a papain derivative with an estimated isoelectric point of 4–5 (Sluyterman & de Graaf, 1972), yields a pK of 4.85 ($\Gamma/2$ = 0.3, 25 °C) in fluorometric titrations (Table I). Although the fluorometrically determined pK value comes closer to that expected for a normal histidyl side chain (pK = 6.4; Tanford & Roxby, 1972) as the effect of the charge on the protein is reduced, the estimated pK value for an uncharged methylthio derivative is still about 1.3–1.6 pK units below that expected for a normal histidyl residue.

The most direct method of observing the ionization of histidyl residues is currently proton NMR spectroscopy. NMR studies of papain derivatives, however, are limited to a narrow range of temperatures. For this reason we have employed fluorescence spectroscopy of papain and papain derivatives to determine the ionization behavior of His-159 as a function of temperature. Examination of Table I reveals that (except at an ionic strength ≤0.01) this pK value, as well as that of His-159 in succinyl-papain, is essentially independent of temperature. This behavior indicates a near-zero heat of ionization for His-159 in the methylthio derivatives and contrasts sharply with the heat of ionization of 7–9 kcal/mol expected for a normal histidyl residue (Izatt & Christensen, 1968).

The change in pK with temperature for His-159 in active papain (Table I), however, indicates a normal heat of ionization of 8 kcal/mol.[6] Thus, removal of the methylthio group from Cys-25 causes the heat of ionization of His-159 to increase by about 8 kcal/mol and its pK to increase by about 4 pK units. These large changes in the thermodynamic parameters for ionization of His-159 are attributed to formation of an ion-pair interaction between His-159 and Cys-25 upon removal of the methylthio group.

Discussion

Kirsch and co-workers have raised serious questions about the likelihood that the histidyl residue at the active stie of acyl-papain derivatives has a pK of about 4 and a heat of ionization near zero (Hinkle & Kirsch, 1971; Zannis & Kirsch, 1978). A normal histidyl residue in a protein might be expected to have an enthalpy of ionization of about 8 kcal/mol and a pK of about 6.4 (Izatt & Christensen, 1968; Tanford & Roxby, 1972). Although considerable variability has been observed for the heat of ionization of histidyl residues in proteins (Westmoreland et al., 1975; Cohen, 1969; Markley, 1975), the only report of a zero heat of ionization of a histidyl residue was from Maurel et al. (1975), who found that in 50% (w/v) water/methanol the deacylation rate for trypsin-catalyzed hydrolysis of N-α-benzoyl-L-arginine ethyl ester was modulated by a group with an enthalpy of ionization of zero ascribed to the His-46 residue of the charge-relay system at the active site of trypsin (see also Maurel & Douzou, 1975).

The possible dependence of pK's as well as heats of ionization on microenvironments, as illustrated by the variability in the enthalpy of ionization of histidyl residues, makes it difficult to predict thermodynamic parameters for the ionization of groups in proteins [see also Knowles (1976)]. There have been reported, however, correlations between enthalpies and free energies for the ionization of simple acids and bases. Zannis & Kirsch (1978) have argued that, on the basis of the relationship between the enthalpy and free energy in substituted anilinium ions (Liotta et al., 1973), a pK = 4 histidyl residue should have a heat of ionization of at least 5 kcal/mol. It was therefore concluded that a zero enthalpy of ionization is unreasonable for His-159 in papain-S-SCH$_3$. It should be noted, however, that in the anilinium ion model system, free

[5] This approximation of charge was based on the presence of 24 cationic arginyl, histidyl, lysyl, and amino-terminal side chains and the presence of six aspartyl residues (assumed pK = 4.0), seven glutamyl residues (assumed pK = 4.5), and one C-terminal carboxyl group (assumed pK = 3.6). The assumed pK values are the intrinsic pK values for these side chains which were used by Tanford & Roxby (1972) to calculate the titration curve of lysozyme. The actual pK values for these groups in a positively charged protein would be expected to be somewhat less (Tanford & Roxby, 1972), so that the estimate of 16–21 for z is probably too high. Assuming that all the carboxyl side chains are deprotonated, one obtains a lower limit of 10 for z.

[6] Values are calculated from the equation $\Delta H = 2.303R(pK_2 - pK_1)/[(1/T_2) - (1/T_1)]$ and standard errors are calculated from the equation SE $\Delta H = 2.303R[(1/T_2) - (1/T_1)]^{-1}(\text{SE } pK_1^2 + \text{SE } pK_2^2)^{1/2}$

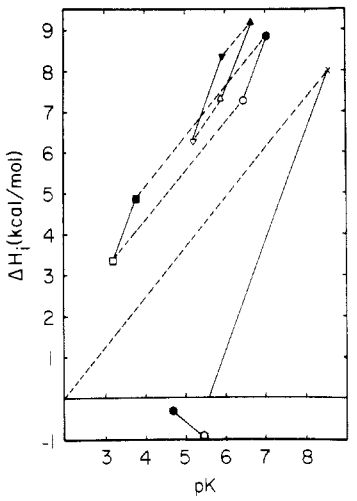

FIGURE 3: Perturbations in enthalpy of ionization and pK (at 25 °C) caused by substituent effects and changes in the medium. The filled symbols represent values for water, the open ones represent values for 50% (w/v) water/methanol: (circles) imidazole; (squares) 4-(5)-bromoimidazole; (triangles) 2,6-lutidine; (inverted triangles) 2-picoline; (hexagons) acetic acid. The × represents the coordinates for His-159 in native papain. The dashed line depicts a substituent effect whereas the solid line depicts the effect of changing the medium.

energies of ionization are altered by electronic substituent effects, whereas the perturbation in the pK for ionization of His-159 is most likely due to its microenvironment.[7] It is conceivable, therefore, that the enthalpy-free energy relationship for His-159 in papain is different from that observed for anilinium ions.

In order to determine whether the enthalpy-free energy relationship generated by altering the local environment of an ionizable group is different from that generated by altering electronic substituents, we undertook a study of the temperature dependence of the ionization constant of imidazole, 4(5)-bromoimidazole, 2-picoline, 2,6-lutidine, and acetic acid in water and in 50% (w/v) water/methanol. pK measurements were obtained at two temperatures on partially neutralized solutions of these substances.

The reported pK values in water and 50% (w/v) water/methanol are averages of values calculated from eq 12 at three degrees of protonation (f_H)

$$pK = pa_H^* + \log f_H/(1 - f_H) \qquad (12)$$

These pK values and the corresponding heats of ionization as determined from the temperature dependence of the pK values are plotted in Figure 3. The parallelographic arrangement of the coordinates for the imidazole and the pyridine bases in water and 50% (w/v) water/methanol which is depicted in Figure 3 suggests that two sets of parallel lines describe the enthalpy–free-energy interactions. For imidazole and 4(5)-bromoimidazole, one set with a slope of 1.2 represents the substituent effect, and the other set with a slope of 2.7 describes the influence of the change in the solvent. Interestingly, the two pyridine and imidazole bases are roughly similar with respect to the changes in pK and enthalpy of ionization that they exhibit in response to solvent and substituent effects in spite of probable differences in the solvation of 2-picoline and 2,6-lutidine. However, the changes in pK and heat of ionization of acetic acid upon going from water to 50% water/methanol are much different from that observed for the nitrogen bases (Figure 3).

Lines with slopes of 1.2 and 2.7 have been drawn in Figure 3 through the enthalpy–free-energy coordinate for the His-159 imidazolyl group of active papain (pK = 8.55, $\Delta H_i°$ = 8.0 kcal/mol at 25 °C). The intersection of those lines with the line defined by $\Delta H_i°$ = 0 indicates that if the decrease in the pK of His-159 in going to papain-S-SCH$_3$ were caused by perturbations resembling electronic substituent effects or changes in the solvent composition or a combination of both, then a pK anywhere between 2 and 5.5 could occur with an enthalpy of ionization of zero. Thus, His-159 could easily have a pK of 4 and a near-zero heat of ionization in thiol-blocked derivatives of papain.

A second objection that has been raised against the assignment of His-159 as the pK = 4 general base catalyst in the deacylation step of papain catalysis is the apparent disparity between the substituent effects for the hydrolysis of substituted benzoyl-papain derivatives and those expected for catalysis by a pK = 4 imidazolyl group at the active site (Zannis & Kirsch, 1978). Zannis and Kirsch found a linear relationship with a slope of 1.02 ± 0.08 between the log k_3 values for deacylation of benzoyl-papain derivatives and log k_3 values found in a variety of studies of the deacylation of benzoyl-chymotrypsin derivatives. These investigators interpreted the slope of unity to indicate that the general base for deacylation of benzoyl-papain intermediates was of the same −1 formal charge as the general base for the deacylation of benzoyl-chymotropsin intermediates.

One fundamental problem with this approach is the assumption that the Asp-102 carboxylate anion of the Asp-102–His-57 charge-relay system of acyl-chymotrypsins acts as the ultimate destination for the proton abstracted from water during general base catalyzed deacylation, i.e., that the participation of the Asp-102 carboxyl group produces a net charge of −1 in the transition state for deacylation. However, Bachovchin & Roberts (1978) have shown by ^{15}N NMR titrations that in the α-lytic protease of Myxobacter, there is no more than 10% transfer of charge from the protonated imidazolium of the histidyl residue to the carboxylate anion in the charge-relay system in the absence of substrate. It may be argued, of course, that the charge-relay system of chymotrypsin behaves in an entirely different manner or that the presence of substrate changes the relative acidity of the histidyl and aspartyl residues. However, proton inventory analysis of solvent deuterium isotope effects in the hydrolysis of acyl-enzyme intermediates of chymotrypsin and other serine proteinases suggests that concerted transfer of a proton from water via His-57 to Asp-102 does not occur with simple substrates (Schowen, 1977). The deacylation of benzoyl-chymotrypsin, therefore, most likely proceeds via general base catalysis by the neutral imidazolyl group of His-57. That appearing to be the case, attempts to demonstrate that the transition state in the deacylation of benzoyl-papain has a full negative charge because it exhibits the same substituent effects as benzoyl-chymtrypsin seem questionable.

In addition to comparing the hydrolysis of substituted benzoyl-papain and chymotrypsin derivatives, Zannis & Kirsch have compared the hydrolysis of substituted benzoyl-papain derivatives and the hydrolysis of low molecular weight benzoate esters in simple model systems. These investigators have argued that since the reactions of anionic nucleophilies with substituted benzoate esters are characterized by ρ values ≥ 2, whereas reactions of neutral nucleophiles with benzoate esters are characterized by ρ values ≤ 1.4 (Hubbard & Kirsch, 1972,

[7] In the native enzyme at physiological pH values, His-159 and Cys-25 form an imidazolium–thiolate ion pair (Johnson et al., 1981; Lewis et al., 1976, 1981), so that on a microscopic scale the environment around His-159 is probably drastically altered upon removal of the full negative charge on the thiolate anion 3 Å from the imidazolium cation.

Table II: ρ Values for the Reactions of Nucleophiles with Substituted Benzoate Esters

nucleophiles	leaving group		
	p-NO_2Ph	2,4-diNO_2Ph	p-ClPh
chymotrypsin[a]	0.97 ± 0.11	1.6 ± 0.3	
imidazole	1.19[b]	1.73 ± 0.07[a]	
imidazole (GB)[c]	1.57[b]		
NH_3[d]	1.43 ± 0.02		1.08 ± 0.12
NH_3 (GB)[c]			1.88 ± 0.01[d]
$(CH_3)_2NOH$[a]	1.28		
N_3[a]	1.8		
OH[a]	2.01 ± 0.01	2.20 ± 0.09	

[a] From Hubbard & Kirsch (1972). [b] From Caplow & Jencks (1962). [c] These ρ values are for general-base (GB) catalysis of nucleophilic attack by a second molecule of the indicated nucleophile. [d] From Kirsch & Kline (1969).

and references cited therein), the ρ value of 2.73 ± 0.32 measured for deacylation of substituted benzoyl-papain derivatives indicates that the catalytic group of the enzyme must be anionic. The data used to support this conclusion along with ρ values for a few other reactions are presented in Table II. Examination of the data in Table II reveals that ρ values for the hydrolysis of substituted benzoate esters increase with the acidity of the leaving group. For example, the ρ values for the imidazole-catalyzed hydrolysis of p-nitrophenyl and 2,4-dinitrophenyl benzoate are 1.2 (Caplow & Jencks, 1962) and 1.7 (Hubbard & Kirsch, 1972), respectively. The p-nitrophenol and 2,4-dinitrophenol leaving groups have pK's of 7.15 and 3.96, respectively (Weast, 1971). The leaving group in the deacylation of papain is the Cys-25 thiol, which has a pK of about 3.3 (Lewis et al., 1976, 1981; Johnson et al., 1981). It is not known to what extent the low pK of the thiol group might cause the ρ value for deacylation of substituted benzoyl-papain derivatives to assume a ρ value higher than that observed for the dinitrophenyl benzoates.

Another difficulty is that the comparison of ρ values rests on the tacit assumption that the ρ values for nucleophilic and general-base-catalyzed hydrolysis are similar, since ρ values for *nucleophilic* attack on simple benzoate esters are used to ascertain the charge on the catalytic group in the *general-base*-catalyzed deacylation benzoyl-papain derivatives. In two cases for which the second-order terms for general base catalysis of nucleophilic attack on benzoate esters have been evaluated, the reported ρ values are higher for general base catalysis than for direct nucleophilic attack (see Table II).

Finally, and most importantly, it should be noted that the determination of ρ values is itself problematical. There appear to be systematic deviations from linearity in the Hammett plots so that the ρ values reported for each reaction are to some extent dependent upon the nature of the substituents chosen for study. This deviation is minimized in linear-free-energy plots of log k_3 for deacylation of substituted benzoyl-papain derivatives vs. log k for hydrolysis of substituted benzoate esters. The slopes of several such plots are listed in Table III. The data in Table III indicate that depending upon the nature of the leaving group and the catalyst, *uncharged* catalysts can indeed exhibit the same sensitivity to electronic effects as the deacylation of benzoyl-papain derivatives. Thus, there appears to be no basis for excluding the direct involvement of an uncharged histidyl residue in the deacylation step in papain-catlayzed reactions. In fact, the ionization properties of His-159 in papain-S-SCH_3 and the sensitivity of the deacylation of benzoyl-papain derivatives to substituent effects are quite consistent with that expected from the ionization behavior of model compounds and the sensitivity of benzoate ester hydrolysis to substituent effects. These observations coupled with the proximity of His-159 to the active-center cysteinyl residue makes it most reasonable to conclude that His-159 is directly involved in catalysis.

Table III: Values for Slopes of Plots of Log k_3 for Deacylation of Benzoyl-papains vs. Log k for Hydrolysis of Substituted Benzoates

type of benzoates	catalyst	slopes ± SD[a] (no. of points)	source of data
p-nitrophenyl			
	OH Nu	1.13 ± 0.08 (4)	b
	NH_3 Nu	1.56 ± 0.07 (3)	d
	Im Nu	1.91 ± 0.12 (4)	b
	Im GB	1.00 (2)	b
2,4-dinitrophenyl			
	OH Nu	1.08 ± 0.08 (4)	c
	Im Nu	1.13 ± 0.04 (4)	c
p-chlorophenyl			
	NH_3 Nu	1.44 ± 0.38 (3)	d
	NH_3 GB	1.11 ± 0.09 (3)	d

[a] Values of log k_3 from Zannis & Kirsch (1978) were plotted against values of log k from each source of data for all substituents used in both studies. The slope ± SD of each correlation was computed by nonweighted linear regression on the points. Abbreviations used: CT, chymotrypsin; GB, general base catalysis; Im, imidazole; SD, standard deviation; Nu, nucleophilic catalysis. [b] Caplow & Jencks (1962). [c] Hubbard & Kirsch (1972). [d] Kirsch & Kline (1969).

References

Akerlof, G. (1932) *J. Am. Chem. Soc. 54*, 4125–4139.

Bachovchin, W. W., & Roberts, J. D. (1978) *J. Am. Chem. Soc. 100*, 8041–8047.

Bates, R. G. (1964) *Determination of pH. Theory and Practice*, pp 52–54, 189–192, 222–229, Wiley, New York.

Bendall, M. R., & Lowe, G. (1976a) *Eur. J. Biochem. 65*, 481–491.

Bendall, M. R., & Lowe, G. (1976b) *Eur. J. Biochem. 65*, 493–502.

Brubacher, L. J., & Bender, M. L. (1966) *J. Am. Chem. Soc. 88*, 5871–5879.

Caplow, M., & Jencks, W. P. (1962) *Biochemistry 1*, 883–893.

Cohen, J. S. (1969) *Nature (London) 223*, 43–46.

Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, pp 282 ff, 514, Academic Press, New York.

Hinkle, P. M., & Kirsch, J. F. (1970) *Biochemistry 9*, 4633–4643.

Hinkle, P. M., & Kirsch, J. F. (1971) *Biochemistry 10*, 3700–3707.

Hubbard, C. D., & Kirsch, J. F. (1972) *Biochemistry 11*, 2483–2493.

Izatt, R. M., & Christensen, J. J. (1968) in *Handbook of Biochemistry. Selected Data for Molecular Biology* (Sober, H. A., Ed.) pp J-49–J-139, Chemical Rubber Company, Cleveland, OH.

Johnson, F. A., Lewis, S. D., & Shafer, J. A. (1981) *Biochemistry* (first of three papers in this issue).

Jolley, C. J., & Yankeelov, J. A., Jr. (1972) *Biochemistry 11*, 164–169.

Kirsch, J. F., & Kline, A. (1969) *J. Am. Chem. Soc. 91*, 1841–1847.

Knowles, J. P. (1976) *CRC Crit. Rev. Biochem. 4*, 165–173.

Lewis, S. D., Johnson, F. A., & Shafer, J. A. (1976) *Biochemistry 15*, 5009–5017.

Lewis, S. D., Johnson, F. A., Ohno, A. K., & Shafer, J. A. (1978) *J. Biol. Chem. 253*, 5080–5086.

Lewis, S. D., Johnson, F. A., & Shafer, J. A. (1981) *Biochemistry* (preceding paper in this issue).

Liotta, C. L., Pardee, E. M., & Hopkins, H. P., Jr. (1973) *J. Am. Chem. Soc. 95*, 2439-2445.
Lowe, G. (1970) *Philos. Trans. R. Soc. London, Ser. B 257*, 237-248.
Lowe, G., & Whitworth, A. S. (1974) *Biochem. J. 141*, 503-515.
Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70-80.
Maurel, P., & Douzou, P. (1975) *J. Biol. Chem. 250*, 2678-2680.
Maurel, P., Hui Bon Boa, G., & Douzou, P. (1975) *J. Biol. Chem. 250*, 1376-1382.
Mitchel, R. E., Chaiken, I. M., & Smith, E. L. (1970) *J. Biol. Chem. 245*, 3485-3492.
Polgar, L. (1973) *Eur. J. Biochem. 33*, 104-109.
Schowen, R. L. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 64-99, Baltimore, MD.
Shedlovsky, T., & Kay, R. L. (1956) *J. Phys. Chem. 60*, 151-155.
Sluyterman, L. A. AE., & de Graaf, M. J. M. (1970) *Biochim. Biophys. Acta 200*, 595-597.
Sluyterman, L. A. AE., & de Graaf, M. J. M. (1972) *Biochim. Biophys. Acta 258*, 554-561.
Sluyterman, L. A. AE., & Wijdenes, J. (1973) *Biochim. Biophys. Acta 302*, 95-101.
Sluyterman, L. A. AE., & Wijdenes, J. (1976) *Eur. J. Biochem. 71*, 383-391.
Tanford, C., & Roxby, R. (1972) *Biochemistry 11*, 2192-2198.
Van Uitert, L. G., & Haas, C. G. (1953) *J. Am. Chem. Soc. 75*, 451-455.
Weast, R. C., Ed. (1971) *Handbook of Chemistry and Physics*, 52nd ed., p D-120, Chemical Rubber Company, Cleveland, OH.
Westmoreland, D. G., Matthews, C. R., Hayes, M. B., & Cohen, J. S. (1975) *J. Biol. Chem. 250*, 7456-7460.
Whitaker, J. R., & Bender, M. L. (1965) *J. Am. Chem. Soc. 87*, 2728-2737.
Williams, A., Lucas, E. C., & Rimmer, A. R. (1972) *J. Chem. Soc., Perkin Trans. 2*, 621-627.
Wolf, A. V., Brown, M. G., & Prentiss, P. G. (1971) in *Handbook of Chemistry and Physics* (Weast, R. C., Ed.) 52nd ed., p D-198, Chemical Rubber Company, Cleveland, OH.
Zannis, V. I., & Kirsch, J. F. (1978) *Biochemistry 17*, 2669-2674.

Nuclear Magnetic Resonance Studies of *Rhodospirillum rubrum* Cytochrome *c'*†

Mark H. Emptage,‡ Antonio V. Xavier,§ John M. Wood,* Basim M. Alsaadi, Geoffrey R. Moore, Robert C. Pitt, Robert J. P. Williams,‖ Richard P. Ambler, and Robert G. Bartsch

ABSTRACT: Cytochrome *c'* from *Rhodospirillum rubrum* has been studied by proton magnetic resonance (NMR) at 270 MHz. The pH and temperature-dependence properties as well as proton water relaxation enhancement and bulk susceptibility measurements were examined. We conclude that the fifth ligand to the iron is histidine. The pH-dependent shift of the heme methyl resonances of the ferric protein shows pK_a's at 5.8 and 8.7. The low-pH equilibrium causes only minor changes in the properties of the protein. However, the high-pH equilibrium causes large changes throughout the NMR spectra which correlate with the reported visible spectral changes. These NMR spectral changes are compared with the low-temperature EPR and Mössbauer spectroscopic data. Analyses of the NMR data show that a second histidine, which is present in the sequence of *c'* from *R. rubrum* but is not conserved in other cytochromes *c'*, is not a "distal" histidine. The nature of the sixth ligand and the significance of the high-pH transition are discussed.

The cytochromes *c'* are an anomalous group of heme proteins whose precise biological function has not yet been established, although it has been suggested that they operate in electron-transport chains (Horio & Kamen, 1970; Kakuno et al., 1973; Lemberg & Barrett, 1973). These proteins are placed in the cytochrome *c'* class of heme proteins by virtue of their possession of a heme group covalently bound to the protein via thioether linkages and the high-spin nature of the heme iron. The sequence of nine cytochromes *c'* have been determined, and all possess heme binding units (Cys-x-y-Cys-His)[1] located near the C-terminus (Ambler, 1973; Meyer et al., 1975; Ambler et al., 1979a,b; R. P. Ambler, unpublished data). By analogy with other types of cytochromes *c*, the conserved histidine residue in this sequence probably provides the fifth ligand to the heme iron. The nature of the sixth ligand is not known. We shall address ourselves to the problem of the

† From the Gray Freshwater Biological Institute (M.H.E., A.V.X., and J.M.W.), University of Minnesota, Navarre, Minnesota 55392, the Inorganic Chemistry Laboratory (B.M.A., G.R.M., R.C.P., and R.J.P.W.), South Parks Road, Oxford OX1 3QR, England, the Department of Molecular Biology (R.P.A.), Kings Building, Mayfield Road, Edinburgh EH9 3JR, Scotland, and the Department of Chemistry (R.G.B.), University of California at San Diego, La Jolla, California 92093. *Received July 13, 1979; revised manuscript received September 2, 1980.* A preliminary report on part of this work has been presented (Emptage et al., 1978). This work was supported in part by grants from the U.S. Public Health Service (1RO1-GM25879) and the National Science Foundation (PCM 7820461).

‡ Present address: Institute for Enzyme Research, University of Wisconsin, Madison, WI 53706.
§ On leave from Centro de Quimica Estrutural, Complexo Interdisciplinar Instituto Superior Tecnico, 1000 Lisboa, Portugal.
‖ A member of the Oxford Enzyme Group.

[1] Abbreviations used: EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; IR, infrared; RHP, *Rhodospirillum* heme protein.

0006-2960/81/0420-0058$01.00/0