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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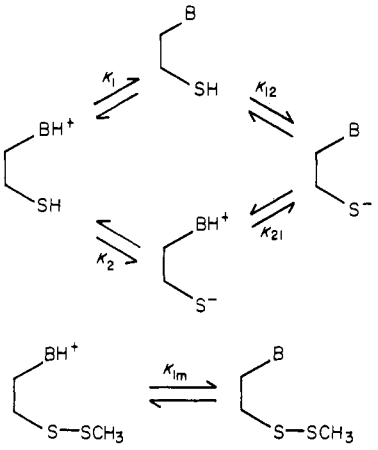
was presumed to be His-159. The model proposed to account for the interactive ionization of Cys-25 and His-159 is illustrated in eq 1 and 2. If $pK_1 = pK_{1m}$, then an analysis of the



(1)

(2)

pH dependence of the difference in proton content of papain and papain-*S*-SCH_3 in terms of the model results in the following values for the four microscopic ionization constants: $pK_{1m} = pK_1 = 4.26$, $pK_2 = 3.34$, $pK_{12} = 7.55$, and $pK_{2i} = 8.47$ at 29 °C and an ionic strength of 0.05. The values of these constants indicate that at physiological pH values Cys-25 and His-159 exist predominantly as an ion pair and that upon neutralization of the negative charge on the thiolate anion (e.g., by protonation or methylthiolation) the p*K* of His-159 drops from about 8.5 to 4.3 (at $\Gamma/2$ 0.05, 29 °C in H_2O). In the preceding paper, we showed that in papain-*S*-SCH_3 (where in Cys-25 is methylthiolated and cannot form a thiolate anion) the p*K* of His-159 is 3.9 ± 0.1 (at $\Gamma/2$ 0.05, 45 °C in ^2H_2O). This value is in tolerable agreement with the value of 4.3 ± 0.1 for the p*K* of His-159 in papain-*S*-SCH_3 determined from the potentiometric difference titrations under somewhat different conditions.[2] If the model depicted in eq 1 and 2 is correct, the p*K* of His-159 should rise to about 8.5 when the methylthio blocking group is removed and Cys-25 forms a thiolate anion. Unfortunately, the decreased solubility of papain at pH values within about 1.5 pH units of its isoelectric point of 9.6 made it impractical to use proton NMR spectroscopy to determine the effect of Cys-25 on the ionization of His-159. In order to circumvent this problem the isoelectric point of papain was shifted by succinylation. Sluyterman & de Graaf (1972) described the preparation of a catalytically active succinyl-papain derivative which has an isoelectric point of 4.3. We have found that this derivative of papain is sufficiently soluble so that the pH dependence of its proton NMR spectrum between pH 6 and 10 could be measured. In this work we report that such measurements indicate that the model illustrated in eq 1 and 2 correctly describes the effect of Cys-25 on the ionization of His-159.

Experimental Procedures

Succinyl-papain-*S*-SCH_3 was prepared from mercuric succinyl-papain. The mercuric succinyl-papain (800 mg) was prepared by scaling up the procedure of Sluyterman & de Graaf (1972). After succinylation, active succinyl-papain was separated from the reaction mixture by activating the mercuric derivative with 11 mM EDTA and 36 mM 2-mercaptoethanol followed by gel filtration through a column of Sephadex G-25 using a 0.05 M pyrophosphate–20 mM EDTA pH 8 buffer as the eluant. The large protein peak eluted at the void volume and was treated with an equimolar quantity of DTT to ensure maximal activation. Succinyl-papain-*S*-SCH_3 was then formed by reacting the protein with a 3.8-fold molar excess of 0.1 M methyl methanethiosulfonate. Succinyl-papain-*S*-SCH_3 in water was concentrated to 20 mg/mL by ultrafiltration and precipitated from solution by the addition of a pH 4 1 M sodium acetate solution to the protein until the mixture had reached pH 4.4. After being cooled at 4 °C for 3 h, the suspension was centrifuged at 20000*g* for 10 min. The precipitate was redissolved in pH 7 0.1 M phosphate in ^2H_2O and the resulting solution filtered through a 0.45-μm Millipore membrane. Seven cycles of ultrafiltration and redilution with 1 volume of ^2H_2O resulted in more than 99.5% deuterium enrichment. When the desired extent of isotope exchange as measured by integration of the HO^2H peak in a sample of filtrate (using a Varian T-60 spectrometer) was reached, the solution was concentrated to 2.5 to 3.0% protein by ultrafiltration. Upon activation, a sample of the succinyl-papain-*S*-SCH_3 exhibited a specific activity toward *N*-α-benzoyl-L-arginine *p*-nitroanilide at pH 6.5 which was twice that of unmodified native papain. This enhanced activity is probably due to a decreased K_m of succinyl-papain for the cationic substrate (see Löffler & Schneder, 1972).

NMR spectra were measured at 150 MHz on a Nicolet NT-150 spectrometer at the Purdue University Biochemical Magnetic Resonance Laboratories. Samples of protein for NMR spectra were in deuterium oxide solutions of Tris and glycine buffers at 45 °C and an ionic strength of 0.05. After the NMR spectrum of a sample of succinyl-papain-*S*-SCH_3 was recorded, the sample was removed from the NMR tube and activated with a 2.5-fold molar excess of dithiothreitol. The pH of the sample solution was then readjusted to its pH value prior to activation, and the NMR spectrum of the resulting solution of succinyl-papain was measured. Acceptable spectra could be obtained after 100–500 pulses. Control experiments indicated little loss of catalytic activity (<10%) under conditions of NMR measurements. Back-titration of a sample of activated succinyl-papain from pH 9.2 to pH 6.8 (by slow addition of 1 M acetic acid at 25 °C) indicated that the pH-dependent chemical shift of the histidyl resonance was due to reversible acid–base equilibria and not irreversible denaturation of the protein. Other materials and procedures used in this work have been described by Johnson et al., (1981).

Results

Spectra of succinyl-papain-*S*-SCH_3 and succinyl-papain at several pH values are depicted in Figure 1. Comparison of the spectra in Figure 1 indicates that removal of the methylthio group from Cys-25 results in the disapperance of the H1′ peak at about 7.68 ppm and the appearance of the peak H1A, whose chemical shift is pH dependent in the pH range 6–10.

The chemical shift of the H1′ peak in the spectra of succinyl-papain-*S*-SCH_3 is essentially identical with the chemical shift observed for the C^{ϵ}_1 H resonance for the unprotonated form of His-159 in papain-*S*-SCH_3 (Johnson et al., 1981), and it is therefore most reasonable to assign the H1′ peak to the C^{ϵ}_1 H resonance of the unprotonated form of His-159 in succinyl-papain-*S*-SCH_3. The pH dependence of the H1A peak which appears upon removal of the blocking group is depicted in Figure 2. The high- and low-pH plateaus of the chemical shifts for this peak are within experimental error of

[2] The proton NMR titrations were carried out in acetate buffers ($\Gamma/2$ 0.05) at 45 °C in deuterium oxide solutions containing 1–1.3 mM protein. The potentiometric titrations were carried out in KCl solutions ($\Gamma/2$ 0.05) at 29 °C in H_2O solutions containing 0.05–0.1 mM protein. The differences in these conditions together with experimental error could account for the difference between the two p*K* values.

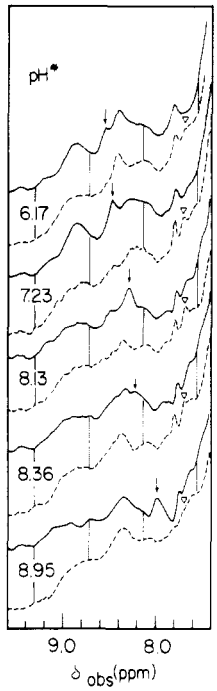


FIGURE 1: NMR spectra of succinyl-papain-*S*-SCH_3 (dashed line) and active succinyl-papain (solid line) at a few pH values. The arrows and triangles denote the chemical shifts of the H1A and H1′ peaks, respectively.

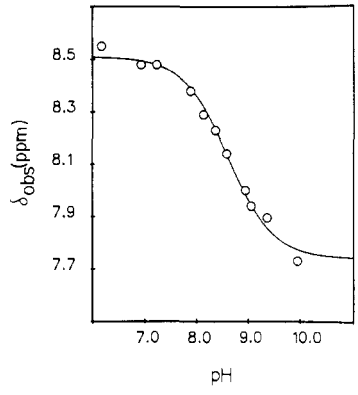


FIGURE 2: pH dependence of the chemical shift of the H1A peak in active succinyl-papain. The solid line represents the best fit of the equation $\delta_{obsd} = [\delta_{H^\circ} + 10^{(pK-pH^*)}\delta_{H^+}/[1 + 10^{(pK-pH^*)}]$ to the pH dependence of the chemical shift (δ_{obsd}) for the H1A peak. The fit to the data yielded the values $pK = 8.62 \pm 0.06$, $\delta_{H^\circ} = 7.74 \pm 0.03$, and $\delta_{H^+} = 8.52 \pm 0.05$.

the values observed for the C^{ϵ_1} H resonance of the protonated and unprotonated forms of His-159 in papain-*S*-SCH_3 (Johnson et al., 1981), suggesting that the pH dependence of the H1A peak reflects the ionization behavior of His-159. Interestingly, removal of the thiol blocking group from succinyl-papain-*S*-SCH_3 at pH 6–7 causes the resonance attributed to the C^{ϵ_1} H of His-159 to move from a position characteristic of the unprotonated form of His-159 to one characteristic of the protonated form, as predicted by the pK's for the equilibria depicted in eq 1.

The pH dependence of the H1A peak depicted in Figure 2 yielded a value of 8.62 ± 0.06 for the p*K* of His-159 in succinyl-papain at 45 °C. This value is consistent with the p*K* value of 8.47 for ionization of His-159 in papain (at 29 °C) that was estimated from an analysis of pH dependence of the difference in proton content of papain and papain-*S*-SCH_3 according to the model of Lewis et al. (1976) depicted in eq 1.

Discussion

The proton NMR studies reported here provide compelling evidence that in succinyl-papain (and by analogy in papain), the p*K* of His-159 is between 8 and 9. This finding verifies the ion-pair model in eq 1 as the proper rationale for accounting for the pH dependence of the difference in proton content of papain and papain-*S*-SCH_3 as determined by potentiometric difference titrations (Lewis et al., 1976). Regardless of the model, the pH dependence of the difference in proton content indicates that on going from papain-*S*-SCH_3 to papain a p*K* of 4.3 ionization is lost and two new ionizations with p*K*'s of 3.3 and 8.5 arise. Since the NMR titrations of papain-*S*-SCH_3 and active succinyl-papain[3] indicate that it is the p*K* of His-159 which shifts from about 4 to 8.5 upon removal of the methylthio group, it is most reasonable to conclude that the remaining $pK = 3.3$ ionization which arises upon removal of the blocking group reflects for the most part ionization of the thiol group of Cys-25. The NMR titrations rule out alternate explanations for the difference in the ionization behavior of papain-*S*-SCH_3 and papain in which the p*K* of Cys-25 is 8.5 and the p*K* of His-159 shifts from 4.3 in papain-*S*-SCH_3 to 3.3 in papain.

The values of the microscopic ionization constants for the equilibria depicted in eq 1 indicate that the presence of the negative charge on the thiolate anion causes a 4.2-unit increase in the p*K* of His-159, and in a reciprocal manner the presence of the positive charge on His-159 is responsible for a 4.2-unit downward perturbation in the p*K* of the active-site thiol group. We have previously shown (Lewis et al., 1976) that these large perturbations in p*K* can reasonably be ascribed to electrostatic interactions between His-159 and Cys-25. Nevertheless, further studies are required before we can fully account for the low p*K* of His-159 in papain-*S*-SCH_3 and the unusual stability of the ion pair in papain. Since the ion pair is the predominant form of the active-site histidyl and cysteinyl residues at physiological pH values, it is reasonable to consider the possibility that this form of the enzyme is the catalytically competent tautomer. The pH–rate profile for papain catalysis reflects ionizations with p*K*'s of 3.2, 3.9, and 8.4 at Γ/2 0.05, 25 °C (Lewis et al., 1978). It is our contention that these macroscopic ionization constants predominantly reflect ionization of Cys-25 ($pK = 3.2$), Asp-158 ($pK = 3.9$), and His-159 ($pK = 8.4$). The pH–rate profile indicates that the active form of the enzyme is one in which one group is protonated and two groups are unprotonated. The predominant form of the enzyme in this protonation state would be one in which Asp-158 is deprotonated while Cys-25 and His-159 exist as an ion pair. The other two doubly deprotonated forms of the catalytic triad exist in much lower concentrations than the one containing the ion pair, but their low concentration does not per se preclude one of these minor forms from being catalytically important. The predominant form, however, is the most attractive candidate as the catalytically competent form of the enzyme. In this form the thiol group exists in its nucleophilic anionic form so that it can efficiently attack the carbonyl carbon atom of the substrate and the protonated imidazolyl group is in a form which allows it to participate as an acid catalyst by protonating the leaving group.

The imidazolium–thiolate ion pair at the active site of papain appears to be an interesting solution to some of the problems inherent in the design of catalysts for displacement reactions at carbonyl carbon atoms in aqueous solution. It is well es-

[3] The low solubility of papain above pH 8 did not permit NMR titrations of active papain.

tablished from the classical studies of Jencks and his coworkers, and others (e.g., Hupe & Jencks, 1977), that the nucleophilic reactivity of thiolate anions toward esters and presumably also amides should increase with the basicity of the thiolate anion.[4] At physiological pH values, however, basic thiolate anions will be predominately in an unreactive protonated form. A similar problem arises in the design of a general acid catalyst for a nucleophilic displacement reaction in that the efficiency of an acid catalyst increases with its acidity, and yet a substantial amount of the acid must be in its protonated form for it to be catalytically effective.

For simple acids and bases the relationships between acidity, nucleophilic reactivity, and catalytic efficiency are usually such that the optimum nucleophile and catalyst will have pK's within one pK unit of the pH at which the reaction is carried out so that to some extent the maximum catalytic effect is limited by the pH of the reaction. These limitations might not hold for the ion-pair system in papain, since the reactivity of the ion-pair system toward a carbonyl substrate may not be a simple function of the individual pK's of His-159 and Cys-25.

The decreased negative charge density on the thiolate anion accompanying its attack on the carbonyl carbon atom of the substrate should be accompanied by an increase in the acidity of the imidazolium cation of His-159. If protonation or partial protonation of the leaving group were concerted with attack of the thiolate anion, protonation of the leaving group by the imidazolium cation also could be accompanied by an increased basicity of the thiolate anion. Thus both the basicity of the thiolate anion and the acidity of the imidazolium cation would be increased in the transition state for a concerted reaction so that the nucleophilic reactivity of the thiolate anion and catalytic ability of the imidazolium cation would be greater than that expected from their respective basicity and acidity in the ground state.

On the assumption that the acylation step in catalysis involves formation of a tetrahedral intermediate, a concerted attack of the ion pair would result in formation of a tetrahedral intermediate with a protonated or partially protonated leaving group. Such a tetrahedral intermediate would be capable of forming the acyl-enzyme intermediate. An unprotonated tetrahedral intermediate, on the other hand, would always eliminate the thiolate anion and break down to substrate rather than form the acyl-enzyme intermediate, unless the rate of protonation of the tetrahedral intermediate were comparable to its rate of breakdown to starting material. Cases in which rates of proton transfer limit partitioning of tetrahedral intermediates to products have been observed for simple nonenzymic reactions including intramolecular acyl transfer between an amino and thiol group.[5] It should be noted, however, that the ion-pair interaction is also capable of facilitating formation of a protonated tetrahedral intermediate via a two-step pathway. Initial thiolate attack on substrate to form an unprotonated tetrahedral intermediate would result in removal of the negative charge on the thiolate anion and cause the pK of the neighboring protonated imidazolium cation to decrease from about 8.5 to 4. If the imidazolium cation were prevented from transfering its proton to water during formation of the tetrahedral intermediate, the leaving group would be in a good position to be protonated by His-159 before the unprotonated tetrahedral intermediate could break down to reactants. Thus, ion-pair catalysis via either concerted or sequential attack on the substrate as proposed here would ensure formation of a protonated tetrahedral intermediate capable of decomposing to the acyl-enzyme intermediate.

Acknowledgments

We are grateful to Dr. John Markley, Dr. Jerry Dallas, and Mr. Milo Westler of the Purdue University Biomedical Magnetic Resonance Laboratory for their help in acquainting us with the 150-MHz NMR spectrometer.

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[4] In the case of thiol anions attacking phenyl esters the Brønsted β value for the dependence of the rate on the pK of the thiol is 0.3 when formation of the tetrahedral intermediate is rate determining and 0.8 when decomposition of the tetrahedral intermediate is rate determining (Hupe & Jencks, 1977).

[5] For an in-depth discussion of how rates of proton transfer effect partitioning of tetrahedral intermediates, see Jenck's (1976) review of this subject.