A study of the stabilization of tetrahedral adducts by trypsin and δ -chymotrypsin

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δ -Chymotrypsin has been alkylated by $1\text{-}^{13}\text{C}$ - and $2\text{-}^{13}\text{C}$ -enriched tosylphenylalanylchloromethane. In the intact inhibitor derivative, signals due to the $1\text{-}^{13}\text{C}$ - and $2\text{-}^{13}\text{C}$ -enriched carbon atoms have chemical shifts which titrate from 55.10 to 59.50 p.p.m. and from 99.10 to 103.66 p.p.m. respectively with similar pK_a values of 8.99 and 8.85 respectively. These signals are assigned to a tetrahedral adduct formed between the hydroxy group of serine-195 and the inhibitor. An additional signal at 58.09 p.p.m. and at 204.85 p.p.m. in the $1\text{-}^{13}\text{C}$ - and $2\text{-}^{13}\text{C}$ -enzyme-inhibitor derivatives respectively does not titrate when the pH is changed and it is assigned to alkylated methionine-192. On denaturation/autolysis of the $1\text{-}^{13}\text{C}$ -enriched enzyme-inhibitor derivative these signals associated with the intact inhibitor derivative are no longer detected, and a new signal, which titrates from 56.28 to 54.84 p.p.m. with a pK_a of 5.26, is detected. The titration shift of this signal is assigned to the deprotonation of the imidazolium cation of alkylated histidine-57 in the denatured/autolysed enzyme-inhibitor derivative. Model compounds which form stable hydrates and hemiketals in aqueous solutions have been synthesized. By comparing the ^{13}C titration shifts of these model compounds with those of the ^{13}C enriched trypsin- and δ -chymotrypsin-inhibitor derivatives, we deduce that, in both of the intact enzyme-inhibitor derivatives, the zwitterionic tetrahedral adduct containing the imidazolium cation of histidine-57 and the hemiketal oxyanion predominates at alkaline pH values. It is estimated that in both the trypsin and δ -chymotrypsin-inhibitor derivatives the concentration of this zwitterionic tetrahedral adduct is 10000-fold greater than it would be in water. We conclude that the pK_a of the oxyanion of the hemiketal in the presence of the imidazolium cation of histidine-57 is 7.9 and 8.9 in the trypsin and δ -chymotrypsin-inhibitor derivatives respectively and that the pK_a of the imidazolium cation of histidine-57 is > 7.9 and > 8.9 when the oxyanion is present as its conjugate acid, whereas, when the oxyanion is present, the pK_a of the imidazolium cation is > 11 in both enzyme-inhibitor derivatives. We discuss how these enzymes preferentially stabilize zwitterionic tetrahedral adducts in the intact enzyme-inhibitor derivatives and how they could stabilize similar tetrahedral intermediates during catalysis. It is suggested that substrate binding could raise the pK_a of the imidazolium cation of histidine-57 before tetrahedral-intermediate formation which would explain the enhanced nucleophilicity of the hydroxy group of serine-195. A molecular model and kinetic scheme is presented which explains why this increase in the pK_a values of the imidazolium cation is not manifested in the pK_a values determined from the pH dependence of k_{cat} values and why these pK_a values are generally less than pK_a values determined from the pH dependence of k_{cat}/K_m values.

INTRODUCTION

It is generally accepted that, in reactions catalysed by the serine proteinases, the formation and breakdown of acyl-enzyme intermediates proceeds via a tetrahedral intermediate (Blow, 1976; Fersht, 1985; Fink, 1987). The specific stabilization of the oxyanion of the tetrahedral intermediate by hydrogen-bonding in the 'oxyanion hole' was first inferred from X-ray-crystallographic data (Henderson, 1970). Kinetic studies with thiono substrates (Asboth & Polgár, 1983) and site-directed mutagenesis of the serine proteinases (Bryan *et al.*, 1986; Wells *et al.*, 1986) have shown that hydrogen-bonding to the oxyanion or its conjugate acid is essential for efficient catalysis by the serine proteinases. It has been argued that the serine proteinases should lower the pK_a of the oxyanion (Malthouse *et al.*, 1985) and raise the pK_a of the imidazolium cation of histidine-57 to facilitate the breakdown of tetrahedral intermediates (Komiyama & Bender, 1979; Kossiakoff & Spencer, 1981; Primrose *et al.*, 1985; Finucane *et al.*, 1989). As tetrahedral intermediates do not accumulate during catalysis, transition-state analogues have been used to study their stabilization by the serine proteinases. One major limitation of X-ray-crystallographic studies of transition-state analogues bound to the serine proteinases is that the hydrogen atoms cannot be observed (Kossiakoff & Spencer, 1981). But as the chemical shifts of ^{13}C nuclei are dependent on

the charge state of ionizable groups, ^{13}C n.m.r. can be used to determine the pK_a of ionizing groups (Jardetsky & Roberts, 1981; Malthouse, 1986). Specific substrate-derived chloromethane inhibitors of trypsin (Shaw & Springhorn, 1967; Coggins *et al.*, 1974) and chymotrypsin (Ong *et al.*, 1965; Schoellmann & Shaw, 1963) alkylate N-3 of the imidazole ring of histidine-57 in both enzymes. Using ^{13}C n.m.r. it has been shown that the hydroxy group of serine-195 in both trypsin (Malthouse *et al.*, 1983, 1985; Scott *et al.*, 1986) and α -chymotrypsin (Finucane *et al.*, 1989) adds to the inhibitor chloromethane to form a tetrahedral adduct analogous to the tetrahedral intermediate thought to occur during catalysis. By examining the pH-dependent changes in the chemical shifts of ^{13}C n.m.r. signals from the ^{13}C -enriched carbon atoms of benzyloxycarbonyl (Z)-[$1\text{-}^{13}\text{C}$]Lys-CH₂-trypsin (Primrose *et al.*, 1985) and Z-[$2\text{-}^{13}\text{C}$]Lys-CH₂-trypsin (Malthouse *et al.*, 1983, 1985) it was deduced that the pK_a of the oxyanion of the tetrahedral adduct was lowered, whereas the pK_a of the imidazolium cation of histidine-57 was raised in these enzyme-inhibitor derivatives. However, a major problem in these studies was the lack of an appropriate model compound which would allow us to determine the titration shifts of both C-1 and C-2 of the inhibitors caused by oxyanion formation and deprotonation of the imidazolium cation of histidine-57. In the present study we have synthesized an appropriate model compound, and it is used to assign the titration

Abbreviations used: Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); Z-, benzyloxycarbonyl-.

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shifts in the trypsin- and chymotrypsin-inhibitor derivatives and to assess how decreases in the dielectric constant of the solvent will affect the pK_a values of the oxyanion of a tetrahedral adduct and the imidazolium cation of histidine-57.

Both histidine-57 and methionine-192 of α -chymotrypsin are alkylated by tosyl[2- ^{13}C]phenylalanylchloromethane (Tos-[2- ^{13}C]Phe-CH₂Cl), producing ^{13}C n.m.r. signals at 99 p.p.m. and 204.8 p.p.m. respectively at low pH (Finucane *et al.*, 1989). In the present study we show that similar signals are observed when δ -chymotrypsin is alkylated by Tos-[2- ^{13}C]Phe-CH₂Cl. When δ -chymotrypsin was alkylated by Tos-[1- ^{13}C]Phe-CH₂Cl, two signals due to ^{13}C -enriched carbon atoms were detected at 6.34 T, one at 55.1 p.p.m. and the other at 58.1 p.p.m. (Malthouse & Finucane, 1991). However, in an earlier low-resolution study of the Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin derivative at 1.88 T (Finucane & Malthouse, 1989), these signals were not resolved from each other, and so the pK_a values and titration shifts reported by Finucane & Malthouse (1989) are incorrect. In the present study we have examined the pH-dependent changes in the signals from the intact and denatured Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin derivative at 6.34 T. We show that the oxyanion pK_a is reduced and that the pK_a of the imidazolium cation of histidine-57 is raised in both trypsin and δ -chymotrypsin-chloromethane-inhibitor derivatives. We discuss how the pK_a values of the oxyanion of the tetrahedral adduct and that of the imidazolium cation of histidine-57 are perturbed in the enzyme-chloromethane-inhibitor derivatives, and we discuss how such pK_a perturbations could facilitate the formation and breakdown of tetrahedral intermediates during catalysis by serine proteinases.

MATERIALS AND METHODS

The materials and methods for preparing and assaying Tos-[1- ^{13}C]Phe-CH₂Cl, Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin, Tos-[2- ^{13}C]Phe-CH₂Cl and Tos-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin were as described previously (Finucane *et al.*, 1989; Malthouse & Finucane, 1991).

Synthesis of 3-bromo-1,1,1-trifluoropropanone

This was synthesized by the acid-catalysed bromination of 1,1,1-trifluoropropanone as described by McBee & Burton (1952).

Synthesis of 1-(3',3',3'-trifluoro-2',2'-dihydroxypropanyl)-3-methylimidazolyl bromide [compound (b), Fig. 1]

A 1.6 ml portion of methylimidazole (0.02 mol) was stirred with 0.5 ml of 12.1 M-HCl in 150 ml of acetonitrile at room temperature. A 4.16 ml portion of 3-bromo-1,1,1-trifluoropropanone (0.04 mol) was then added in 0.26 ml aliquots over a period of 2–3 days. On adding 0.6 ml of 12.1 M-HCl, the product crystallized, was filtered, washed with acetonitrile and dried. Yield 1.6 g (28 %) (Found: C, 29.4; H, 3.5; N, 10.0; F 18.6; Br, 31.1. Calc. for C₇H₁₀N₂F₃Br: C, 28.9; H, 3.5; N, 9.6; F, 19.6; Br,

27.5. ^{13}C n.m.r.: chemical shift (δ) (p.p.m.) (water, pH 1.8) 138.3 (C-2), 36.9 (C-3'), 124.0 (C-4), 124.9 (C-5), 52.8 (C-6), 92.0, J_{CF} 32 Hz (C-7), 123.2, J_{CF} 288 Hz (C-8). ^1H n.m.r.: δ (p.p.m.) ([^2H]dimethyl sulphoxide) 3.9 [3H, s, N(CH₃)] 4.46 (2H, s, CH₂), 7.71 (2H, s, CH=CH), 9.14 (1H, s, N-CH-N).

Synthesis of 1-(3',3',3'-trifluoro-2',2'-dihydroxypropanyl)imidazolyl bromide [compound (a), Fig. 1]

This was prepared in the same way as compound (b), except that the molar ratio of imidazole to 3-bromo-1,1,1-trifluoropropanone was 2:1 to prevent the formation of the di-alkylated imidazole derivative. Compound (a) did not crystallize out on acidification. Therefore organic solvent was removed by rotary evaporation and the product was redissolved in water. Analysis by ^{13}C n.m.r. and ^1H n.m.r. showed that this was a mixture of imidazole and compound (a) [3 mol of imidazole/1 mol of compound (a)]. This solution was used for pH titrations. ^{13}C n.m.r.: δ (p.p.m.) (water, pH 2.9) 137.2 (C-2), 120.3 (C-4), 124.7 (C-5), 52.8 (C-6), 92.2 J_{CF} 32 Hz (C-7), 123.4, J_{CF} 288 Hz (C-8). ^1H n.m.r.: δ (p.p.m.) ([^2H]dimethyl sulphoxide) 4.54 (2H, s, CH₂), 7.75 (2H, s, CH=CH), 9.17 (1H, s, N-CH-N).

N.m.r. spectra

^{13}C spectra of Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin and non- ^{13}C -enriched control samples were obtained at 6.34 T using a JEOL JNM GX-270 spectrometer operating at 67.80 MHz for ^{13}C nuclei. The sample size was 2.5–4.0 ml in 10 mm-diameter sample tubes. The samples contained 1.7–2.9 mM-protein, $^2\text{H}_2\text{O}$ (20 %, v/v) to obtain a deuterium lock signal, 0.4 M-KCl to minimize dimerization at alkaline pH values, as well as 50 mM-glycine and 50 mM-glycylglycine to minimize pH changes at alkaline pH values. Spectral conditions were: 4096 time-domain data points; spectral width 260 p.p.m.; 0.116 s acquisition time; 0.084 s relaxation delay time; 7 μs pulse width (14.5 μs = 90° pulse); 40 Hz exponential weighting factor; 9000 transients were recorded per spectrum; composite pulse decoupling was implemented using standard JEOL instrumentation. Sample temperatures were 28 $\pm$ 1 °C. ^{13}C n.m.r. spectra of Tos-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin were determined as described by Finucane *et al.* (1989), except that the sample size was 1–2 ml in 10 mm-diameter sample tubes. Aqueous solutions of the model compounds [compounds (a) and (b); see Results section] were examined in 100 % water, 50 % (w/w) dioxan and 70 % (w/w) methanol. A concentric 4 mm tube containing $^2\text{H}_2\text{O}$ (99.8 atom %) was used to obtain a deuterium lock signal. The sample volumes and concentrations of model compounds used in the pH titrations were 2–2.5 ml and 0.2–2 M respectively. Spectra of compounds (a) or (b) in 70 % (w/w) methanol were obtained at 6.34 T. Spectral conditions were: 16384 time-domain data points; spectral width 143.5 p.p.m.; 0.842 s acquisition time; 0.368 s relaxation delay time; 7.7 μs pulse width; 512 transients were recorded per spectrum; composition pulse decoupling was used, and it was gated off during the relaxation delay time. Spectra of compounds (a) or (b) in 100 % water and 50 % (w/w) dioxan were obtained at 1.88 T using a Bruker WP80 wide-bore spectrometer operating at 20.115 MHz for ^{13}C nuclei. Typical spectral conditions used were: 8192 time-domain data points; spectral width 220 p.p.m.; 0.926 s acquisition time; 0.25 s relaxation delay time; 4 μs pulse width (11 μs = 90° pulse); 4000–64000 transients were recorded per spectrum; 1.2 W broad-band decoupling was used, and it was gated off during the relaxation delay.

^{13}C chemical shifts in fully aqueous media were referenced to tetramethylsilane at 0 p.p.m. as described by Finucane *et al.* (1989). Chemical shifts of 67.4 and 49.9 p.p.m. were used as secondary references when the solvent was 50 % (w/w) dioxan and 70 % (w/w) methanol respectively.

Fig. 1. Structures of model compounds

R = H and R = CH₃ for the hydrate and hemiketal forms of compounds (a) and (b) respectively.

Calculation of pK_a values

The pK_a values of hydroxy groups were calculated by using the relationship:

$$pK_a = -7.39 \Sigma \sigma_i + 15.472 \text{ (De Tar, 1982)}$$

When calculating the pK_a values of the hydroxy groups of hydrates, a statistical factor of $\log 2$ was subtracted from the pK_a value calculated in this way. The pK_1 values (Scheme 2 in the Results section below) of *N*-alkylated imidazolium ions were calculated using the relationship:

$$pK_a = -0.997 \Sigma \sigma_i + 7.3 \text{ (Malthouse et al., 1985)}$$

The pK_a of 7.3 when $\Sigma \sigma_i = 0$ is the pK_a of *N*-ethyl-imidazole (Elguero *et al.*, 1968; Paiva *et al.*, 1976; Primrose *et al.*, 1985), which was incorrectly reported as 7.14 by Malthouse *et al.* (1985). The pK_4 values (Scheme 2 in the Results section below) for *N*-alkylated imidazolium ions were calculated by using the relationship:

$$pK_4 = pK_3 + pK_1 - pK_2$$

A substituent constant (σ_1) of 0.29 was used for the OCH_3 substituent (Exner, 1978). All other substituent constants used were those quoted by Malthouse *et al.* (1985) and Finucane *et al.* (1989).

The effect of charge-charge interactions on pK_a values was estimated by using the relationship:

$$pK_a = 243.47/R \cdot D_E$$

where R and D_E are the distance (in Å; 1 Å $\equiv$ 0.1 nm) and effective dielectric constant respectively between the charges (Bjerrum, 1923; Westheimer & Shookhoff, 1939). When estimating the charge separations in the intact Tos-Phe-CH₂- δ -chymotrypsin and Z-Lys-CH₂-trypsin derivatives we assumed that the small movements of the imidazole group [approx. 0.04 nm (0.4 Å)] and of the hydroxy group [approx. 0.05 nm (0.5 Å)] of serine-195, which have been observed when the intact Z-Lys-CH₂-trypsin derivative is formed (Scott *et al.*, 1986), also occurred when the intact Tos-Phe-CH₂- δ -chymotrypsin derivative is formed. The charge separations used were: oxyanion to imidazolium cation, 0.47 nm (4.7 Å); oxyanion to carboxylate anion, 0.88 nm (8.8 Å); imidazolium cation and carboxylate anion, 0.41 nm (4.1 Å). The oxyanion, imidazolium cation and carboxylate anion are those of the hemiketal hydroxy group (see Schemes 1 and 2 in the Results section below), histidine-57 and aspartate-102 respectively in both enzyme inhibitor derivatives. In all calculations it was assumed that aspartate-102 was fully ionized. The values of D_E used are given in the Results section.

RESULTS AND DISCUSSION

Samples of δ -chymotrypsin were alkylated by Tos-[1-¹³C]Phe-CH₂Cl and Tos-[2-¹³C]Phe-CH₂Cl as described previously (Finucane *et al.*, 1989; Malthouse & Finucane, 1991).

Effect of pH on the ¹³C signals from the intact Tos-[2-¹³C]Phe-CH₂- δ -chymotrypsin derivative and the assignment of these signals

After the alkylation of δ -chymotrypsin by Tos-[2-¹³C]Phe-CH₂Cl two signals were detected which were not present in the ¹³C n.m.r. spectrum of δ -chymotrypsin. One of these signals did not titrate from pH 4.0 to pH 9.0 and it had a chemical shift of 204.85 ± 0.16 p.p.m. The chemical shift of the other signal increased from 99.10 ± 0.02 p.p.m. to 103.66 ± 0.03 p.p.m. as the pH increased, and this titration shift was due to a single ionizing group with a pK_a of 8.85 ± 0.03 . The signal at 204.85 ± 0.16 p.p.m. is essentially the same as the signal at 204.82 ± 0.11 p.p.m. from the intact Tos-[2-¹³C]Phe-CH₂- α -chymotrypsin derivative, which

was assigned (Finucane *et al.*, 1989) to the ¹³C-enriched sp² hybridized inhibitor carbon atom after it has alkylated methionine-192 (Structures A and B in Scheme 1). The chemical shift of the signal at 99.10 to 103.66 p.p.m. is characteristic of an sp³ hybridized carbon atom, and it is very similar to the other signal observed in the intact Tos-[2-¹³C]Phe-CH₂- α -chymotrypsin derivative (Table 1), which was assigned (Finucane *et al.*, 1989) to a hemiketal formed by addition of the hydroxy group of serine-195 to the ¹³C-enriched inhibitor carbonyl carbon (structures A and B in Scheme 1).

Substrate-derived oxomethylene inhibitors of pepsin have been shown to bind to pepsin as hydrates (Rich *et al.*, 1982, 1984; Rich, 1985; James & Sielecki, 1985; Mailbaum *et al.*, 1987). X-ray crystallography has been used to show that the C-2 ketone carbon of the inhibitor is not hydrated in the intact Z-Lys-CH₂-trypsin derivative; instead it forms a hemiketal with the hydroxy group of serine-195 (Scott *et al.*, 1986). The chemical shifts of sp³ hybridized hemiketal and hydrate carbon atoms are similar, except that those of hemiketals are 2–3 p.p.m. larger than those of the corresponding hydrate (Table 2). However, the chemical shifts of the sp³ hybridized hydrate and hemiketal carbon atoms of halomethanes are not significantly perturbed (< 0.6 p.p.m.) when they alkylate imidazole, provided the resulting *N*-alkylated imidazoles are fully protonated (Table 2). Therefore, as the chemical shifts of the intact Tos-[2-¹³C]Phe-CH₂- α -chymotrypsin and Tos-[2-¹³C]Phe-CH₂- δ -chymotrypsin derivatives at low pH are very similar to those of the sp³ hybridized hemiketal carbon atoms of Tos-[2-¹³C]Phe-CH₂Cl (Table 2), this supports our earlier assumption (Finucane *et al.*, 1989) that a hemiketal species is formed in these enzyme-inhibitor derivatives (structures A and B in Scheme 1). Although the possibility that the chemical shifts of quaternary hydrate or hemiketal carbon atoms may be perturbed within the intact enzyme-chloromethane-inhibitor derivatives cannot be dismissed, the relative insensitivity of β -quaternary hydrate carbon atoms to solvent composition (Table 3) makes this possibility unlikely.

Effect of pH on the ¹³C signals from the intact and denatured Tos-[1-¹³C]Phe-CH₂- δ -chymotrypsin derivative and the assignment of these signals

Difference spectra at different pHs (Figs. 2c–2g) were obtained by subtracting control spectra of the unenriched Tos-Phe-CH₂- δ -chymotrypsin derivative (e.g. Fig. 2b) from spectra of the Tos-[1-¹³C]Phe-CH₂- δ -chymotrypsin derivative at the same pH (e.g. Fig. 2a). At pH 3.12 the difference spectrum (Fig. 2c) contained two signals (peaks A and B, Fig. 2c) due to ¹³C-enriched carbon atoms. In an earlier low-resolution study (Finucane & Malthouse, 1989) at 1.88 T these two signals were not resolved, and a single broad peak was observed. From studies of the linewidth of signal A it has been shown (Malthouse & Finucane, 1991) that the enzyme-inhibitor derivative is monomeric at pH 3.3 in the absence of 0.4 M-KCl. However, in order to prevent aggregation at higher pH values, 0.4 M-KCl was included in all samples (Finucane *et al.*, 1989).

The chemical shift of peak A increased with increasing pH, whereas that of peak B stayed constant. Consequently peak B was not resolved from peak A at pH 8.15 (Fig. 2d). After the pH was raised from pH 3.12 (Fig. 2c) to pH 9.9, two spectra were recorded consecutively (Figs. 2e and 2f) and a new signal, peak C, was detected. The intensity of peak C increased with increasing time, and there was a concomitant decrease in the intensity of peak A (Figs. 2e and 2f). When the pH was decreased to pH 3.12, the chemical shift of peak A decreased to its original value, whereas the chemical shift of peak C increased and peak B was no longer detected (Fig. 2g). Since the sample retained 0.45% of its original catalytic activity, we conclude that peak C

Table 1. Titration constants obtained by ^{13}C n.m.r. studies of Tos-Phe-CH₂-chymotrypsin and Z-Lys-CH₂-trypsin derivativesAll chemical shifts have been referenced as described by Finucane *et al.* (1989).

Enzyme	Inhibitor	State of enzyme-inhibitor derivative	Chemical shift (p.p.m.)			pK_a	Reference
			δ_1^*	δ_2^*	$\delta_2 - \delta_1$		
Trypsin	Z-[1- ^{13}C]Lys-CH ₂ Cl	Intact	54.84	59.39	4.55	7.99	Primrose <i>et al.</i> (1985)
δ -Chymotrypsin	Tos-[1- ^{13}C]Phe-CH ₂ Cl		55.10	59.50	4.40	8.99	The present work
Trypsin	Z-[2- ^{13}C]Lys-CH ₂ Cl		98.95	103.08	4.13	7.88	Malthouse <i>et al.</i> (1985)
δ -Chymotrypsin	Tos-[2- ^{13}C]Phe-CH ₂ Cl		99.10	103.66	4.56	8.85	The present work
α -Chymotrypsin	Tos-[2- ^{13}C]Phe-CH ₂ Cl		99.08	103.44	4.36	8.67	Finucane <i>et al.</i> (1989)
α -Chymotrypsin	Tos-[2- ^{13}C]Phe-CH ₂ Cl	Denatured	203.52	206.08	2.56	5.27	Finucane <i>et al.</i> (1989)
Trypsin	Z-[2- ^{13}C]Lys-CH ₂ Cl		203.64	206.62	2.98	5.26	Malthouse <i>et al.</i> (1985)
Trypsin	Z-[2- ^{13}C]Lys-CH ₂ Cl		95.80	96.20	0.40	†	Malthouse <i>et al.</i> (1985)
Trypsin	Z-[1- ^{13}C]Lys-CH ₂ Cl		56.04	54.14	-1.90	5.36	Primrose <i>et al.</i> (1985)
δ -Chymotrypsin	Tos-[1- ^{13}C]Phe-CH ₂ Cl		56.28	54.84	-1.44	5.36	The present work

* δ_1 and δ_2 are the pH-independent chemical shifts at low and high pH respectively.† pK_a was not determined. **Scheme 1.** Structures and chemical shifts of the intact and denatured Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin derivatives

is produced by alkaline denaturation/autolysis and that peak B is due to an alkali-labile species. The results of detailed studies of the pH-dependent changes in the chemical shifts of peaks A, B and C are presented in Fig. 3. The chemical shift of peak A increased with increasing pH, resulting in a positive titration shift of 4.40 p.p.m., which was consistent with a single ionizing group of pK_a 8.99 (●, Fig. 3). This pK_a is similar to that determined for the hemiketal carbon atom of the intact Tos-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin derivative (Table 1) and the titration shift is very similar to that observed for the α -methylene carbon atom of the inhibitor in the intact Z-[1- ^{13}C]Lys-CH₂-trypsin derivative (Table 1). Therefore peak A (55.10–59.50 p.p.m.) is assigned to the α -methylene carbon atom of the intact Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin derivative (structures A and B; Scheme 1). In an earlier low-resolution study (Finucane & Malthouse, 1989) of the Tos-[1- ^{13}C]Phe-CH₂- α -chymotrypsin derivative at 1.88 T, the estimates of the titration shift and pK_a value associated with peak A were incorrect, as it was not resolved from peaks B and C. The slightly lower pK_a associated with the hemiketal carbon atom of the intact Tos-

[2- ^{13}C]Phe-CH₂- α -chymotrypsin derivative may reflect an electrostatic effect and/or a conformational change resulting from the fact that the residues Ser-14 and Arg-15 of δ -chymotrypsin are not present in α -chymotrypsin (Dixon & Webb, 1979). Peak B was not detected after alkaline denaturation/autolysis, and its chemical shift (58.09 ± 0.09 p.p.m.) was independent of pH (▲, Fig. 3). The signals at 204.85 ± 0.16 p.p.m. in the intact Tos-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin derivative and the signal at 204.82 ± 0.11 p.p.m. in the intact Tos-[2- ^{13}C]Phe-CH₂- α -chymotrypsin derivative (Finucane *et al.*, 1989) were also alkali-labile and their chemical shifts were also independent of pH. Therefore, as argued by Finucane *et al.* (1989), these signals, and that of signal B, are assigned to the species resulting from the alkylation of methionine-192 in the intact inhibitor derivative (structures A and B; Scheme 1). The chemical shift of peak C decreased with increasing pH, and its titration shift (-1.44 p.p.m.) was consistent with a single ionizing group of pK_a 5.36 (Fig. 3). This pK_a is very similar to the pK_a values obtained from the ^{13}C -enriched carbon atoms in the denatured Tos-[2- ^{13}C]Phe-CH₂- α -chymotrypsin, Z-[2- ^{13}C]Lys-CH₂-trypsin

Table 2. Chemical shifts (δ) of the sp^3 hybridized quaternary carbon atoms of hydrates and hemiketals in aq. 70% (w/w) methanol

Compound	pH	δ (p.p.m.)		$\Delta\delta^*$ (p.p.m.)
		Hydrate	Hemiketal	
Tos-[2- ^{13}C]Phe-CH ₂ Cl	5.3	96.35	99.20†	2.85
Tos-[2- ^{13}C]Phe-CH ₂ Cl	5.3	96.35	99.68†	3.33
Z-[2- ^{13}C]Lys-CH ₂ Cl	3.1	96.40	†	†
Z-[2- ^{13}C]Lys-CH ₂ -trypsin		95.80§	98.95	3.15
Tos-[2- ^{13}C]Phe-CH ₂ - α -chymotrypsin		†	99.08	†
Tos-[2- ^{13}C]Phe-CH ₂ - δ -chymotrypsin		†	99.10	†
1,1,1-Trifluoro-3-bromoacetone	2.0	92.46	95.23	2.77
Compound (a) (Fig. 1)	1.2	92.25	94.64	2.39
Compound (b) (Fig. 1)	4.7	92.22	95.03	2.81

* Chemical shift of the sp^3 hybridized hemiketal carbon minus the chemical shift of the sp^3 hybridized hydrate carbon.

† As there are two chiral carbon atoms in these hemiketals, two diastereoisomers are formed, and so two hemiketal signals were observed.

‡ Not determined.

§ Hydrate signal in the denatured derivative at low pH.

|| Hemiketal signal in the intact derivative at low pH.

and Z-[1- ^{13}C]Lys-CH₂-trypsin derivatives (Table 1). The negative titration shift of peak C is also similar to that observed for the denatured Z-[1- ^{13}C]Lys-CH₂-trypsin derivative. Such negative titration shifts are characteristic of the α -methylene carbon atom of *N*-alkylated imidazoles when deprotonation of the imidazolium cation has occurred (Primrose *et al.*, 1985). This therefore confirms that the inhibitor has alkylated histidine. Since C-2 of the inhibitor is sp^2 hybridized in the denatured inhibitor derivative (Finucane *et al.*, 1989), peak C is assigned to the α -methylene carbon atom of *N*-3-alkylated histidine-57 in the denatured inhibitor derivative (structures C and D; Scheme 1).

Model compounds

The C-2' carbonyl group of 1-(3',3',3'-trifluoro-2'-propanyl)-imidazole is hydrated [see C-7 of compound (a); Fig. 1] both in 100% water and in aq. 50% (w/w) dioxan solutions as is the C-2' carbonyl group of its *N*-3-methylated derivative [see C-7 of compound (b), Fig. 1]. In aq. 70% (w/w) methanol, both methanol and water add to the C-2' carbonyl groups of compounds (a) and (b) to form hydrates and hemiketals respectively (Fig. 1). These hydrate and hemiketal forms of compounds (a) and (b) are good low- M_r analogues of the tetrahedral adducts which occur in the Z-Lys-CH₂-trypsin and Tos-Phe-CH₂-chymotrypsin derivatives.

Linear free-energy relationships were used (see the Materials and methods section) to calculate the microscopic pK_a values (pK_1 , pK_2 , pK_3 and pK_4) of compound (a) in 100% water. The macroscopic pK_a values [$K_1 = K_1 + K_2$, and $K_{II} = K_3 K_4 / (K_3 + K_4)$] of 6.4 (pK_I) and 9.6 (pK_{II}) calculated from these microscopic pK_a values were in good agreement with those ($pK_I = 6.3$ and $pK_{II} = 9.8$; Scheme 2) determined from the pH-dependence of the chemical shifts (Fig. 3) of the α -methylene [C-6 of compound (a); Fig. 1] and β -quaternary [C-7 of compound (a); Fig. 1] carbon atoms of compound (a) in 100% water (Table 3). If, as predicted by linear free-energy relationships, $K_1 \gg K_2$ and $K_4 \gg K_3$, then, within experimental accuracy, $pK_1 = pK_I$ and $pK_3 = pK_{II}$ (Scheme 2). Therefore the experimental macroscopic pK_a values of 6.3 and 9.8 will reflect the deprotonation of the imidazolium ion (pK_1) and the formation of the oxyanion (pK_3)

Fig. 2. Effect of pH on the ^{13}C n.m.r. spectra of Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin

Acquisition parameters were as described in the Materials and methods section. Sample conditions were: (a) 2.5 ml of 2.9 mM-Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin at pH 3.12; (b) 2.5 ml of 2.9 mM-Tos-Phe-CH₂- δ -chymotrypsin at pH 3.12; (c) difference spectrum, (a) - (b). Spectra (d), (e), (f) and (g) are difference spectra obtained in the same way as spectrum (c), except that the protein concentrations, volumes and pHs were: (d) 2.9 mM, 2.5 ml, pH 8.15; (e) and (f) 2.4 mM, 3.0 ml, pH 9.90; (g) 1.9 mM, 3.8 ml, pH 3.12. Spectra (c), (e), (f) and (g) were obtained sequentially using the same sample. All samples contained 20% (v/v) $^2\text{H}_2\text{O}$, 0.4 M-KCl, 50 mM-glycine and 50 mM-glycylglycine.

respectively (Scheme 2). This assignment is confirmed by the negative titration shift (Fig. 4 and Table 3) of the α -methylene carbon atom, which is dependent on pK_1 , and this is typical of that expected for deprotonation of an imidazolium cation (Primrose *et al.*, 1985). By using the microscopic pK_a values calculated using linear free-energy relationships (Scheme 2), the tautomeric ratio ($K_T = K_1/K_2 = K_3/K_4$; Scheme 2) of the neutral species [species (ii); Scheme 2] of the hydrate form of compound (a) to its zwitterionic form [species (iii); Scheme 2] is 126. Consequently, owing to the low concentration of the zwitterionic form of compound (a), the titration shifts and pK_a values (pK_2 and pK_4 ; Scheme 2) for protonation and deprotonation of the zwitterionic species cannot be experimentally determined. However, the hydrate form of compound (b) is analogous to species (i) and (iii) of the hydrate form of compound (a) (Scheme 2),

Fig. 3. pH titration of signals due to the ^{13}C -enriched carbon atoms in the intact and denatured Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin derivative

Acquisition parameters and sample conditions were as described in the Materials and methods section. ●, Signal A in the intact Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin derivative. The continuous line was calculated by using eqn. (i):

$$\delta_{\text{obs.}} = (S_1 + S_2 \cdot K_a / [\text{H}]) / (1 + K_a / [\text{H}]) \quad (\text{i})$$

and the fitted parameters $\text{p}K_a = 8.99 \pm 0.06$, $S_1 = 55.10 \pm 0.02$ p.p.m. and $S_2 = 59.50 \pm 0.05$ p.p.m. ▲, Signal B in the intact Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin derivative. The continuous line is the mean of the data points, which was 58.09 ± 0.09 p.p.m. ■, Signal C in the denatured/autolysed Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin derivative. The continuous line was calculated using eqn. (i) above and the fitted parameters. $\text{p}K_a = 5.36 \pm 0.06$, $S_1 = 56.28 \pm 0.02$ p.p.m. and $S_2 = 54.84 \pm 0.01$ p.p.m.

except that the N-3 proton has been replaced by a methyl group. Therefore, if it is assumed that this modification does not perturb the $\text{p}K_a$ values of the β -hydroxy groups, then the hydrate form of compound (b) can be used to estimate $\text{p}K_2$ of the hydrate form of compound (a) (Scheme 2). The validity of this assumption is supported by the fact that the $\text{p}K_a$ of the hydrate form of compound (b) ($\text{p}K_a = 8.23$; Table 3) in 100% water is in reasonable agreement with that predicted for $\text{p}K_2$ of the hydrate form of compound (a) ($\text{p}K_2 = 8.5$; Scheme 2) using linear free-energy relationships. Thus, using the experimentally determined estimates of the microscopic $\text{p}K_a$ values ($\text{p}K_1 = \text{p}K_1 = 6.3$, $\text{p}K_2 = 8.2$, and $\text{p}K_{11} = \text{p}K_3 = 9.8$; Table 3 and Scheme 2) and the relationship $\text{p}K_4 = \text{p}K_3 + \text{p}K_1 - \text{p}K_2$, we estimate that $\text{p}K_4 = 7.9$ for the hydrate form of compound (a) (Scheme 2). This estimate is in reasonable agreement with the $\text{p}K_4$ 7.5 value calculated by using linear free-energy relationships. As linear free-energy relationships can only be used to estimate $\text{p}K_a$ values in fully aqueous media, we have used the approach outlined above to calculate the microscopic $\text{p}K_a$ values and K_T values of the hydrate and hemiketal forms of compound (a) in mixed solvents (Scheme

2) using the macroscopic $\text{p}K_a$ values of the appropriate forms of compounds (a) and (b) (Table 3).

In all the solvents used, $K_T \gg 1$ (Scheme 2), and so all the titration shifts in Table 3 represent full titration shifts for oxyanion formation and for deprotonation of the imidazolium cation. Therefore deprotonation of the imidazolium cation ($\text{p}K_1$; Scheme 2) of the hemiketal form of compound (a) produces a titration shift of -1.90 p.p.m. and $+0.89$ p.p.m. at the α -methylene and β -hemiketal carbon atoms respectively (Table 3). The α -methylene carbon titration shift is typical of that expected for the α -methylene carbon atom of an *N*-alkylated imidazole (Primrose *et al.*, 1985), whereas the titration shift of the β -hemiketal carbon atom confirms an earlier prediction that such titration shifts would be small (Malthouse *et al.*, 1985). Oxyanion formation when the imidazole group is neutral ($\text{p}K_3$; Scheme 2) produces titration shifts of 2.59 p.p.m. and 3.97 p.p.m. at the α -methylene and β -hemiketal carbon atoms respectively of the hemiketal form of compound (a) (Table 3). Slightly larger titration shifts of 2.87 p.p.m. and 4.38 p.p.m. are observed at the α - and β -carbons of compound (b) where the imidazole group is positively charged (Table 3). Liang & Abeles (1987) observed similar hemiketal-carbon-atom titration shifts of 4.3 and 4.4 p.p.m. in model hemiketals which did not contain a second ionizing group. Finucane *et al.* (1989) stated that the observed titration shifts of two or more interacting groups are not necessarily the same as for either group in isolation. However, from our results (Table 3) it is clear that the neutral or positively charged imidazole moiety does not significantly affect the magnitude of the oxyanion titration shift at the β -hemiketal carbon atom.

The interaction between the oxyanion and imidazolium cation of compound (a) raises the $\text{p}K_a$ of the imidazolium cation ($\text{p}K_4 - \text{p}K_1$) by 1.1–1.6 $\text{p}K_a$ units and it decreases the $\text{p}K_a$ of the oxyanion ($\text{p}K_3 - \text{p}K_2$) by the same amount (Scheme 2). Water, aq. 50% (w/w) dioxan and aq. 70% (w/w) methanol have dielectric constants of 78.5, 34.6 and 45 at 25 °C respectively (Akerlof, 1932; Akerlof & Short, 1936; King & Josephs, 1944). If only charge-charge interactions are considered, then decreasing the dielectric constant should increase the $\text{p}K_a$ values of neutral acids, but it should only have a small or negligible effect on the $\text{p}K_a$ values of cationic acids (Edsall & Wyman, 1958). Therefore the increase in $\text{p}K_3$ and the smaller decrease in $\text{p}K_1$ on decreasing the dielectric constant of the solvent is expected (Scheme 2). A similar decrease in the $\text{p}K_a$ of imidazole when the dielectric constant of the solvent is decreased has been observed by Komiyama *et al.* (1977). When the dielectric constant decreases, $\text{p}K_2$ should increase by an amount less than, or equal to, the increase in $\text{p}K_3$, depending on the degree of charge neutralization in the zwitterionic species [species (iii), Scheme 2] according to classical electrostatic considerations (Edsall & Wyman, 1958). The fact that $\text{p}K_4$ decreased while $\text{p}K_2$ increased more than $\text{p}K_3$ when the solvent dielectric constant was decreased (Scheme 2) is therefore not consistent with such classical electrostatic considerations. These inconsistencies with classical electrostatic theory most probably reflect the fact that such electrostatic interactions can be offset by solvation effects; such solvation effects are discussed below.

Assignment of the titration shifts and $\text{p}K_a$ values associated with the signals from the intact 1- ^{13}C - and 2- ^{13}C -enriched Tos-Phe-CH $_2$ -chymotrypsin and Z-Lys-CH $_2$ -trypsin derivatives

From our studies of model hemiketals (Table 3) we expect deprotonation of the imidazolium cation ($\text{p}K_1$; Scheme 2) to produce a negative titration shift of approx. -1.9 p.p.m. and a small positive titration shift of approx. 0.9 p.p.m. at the α -methylene and β -hemiketal carbon atoms respectively of the

Scheme 2. Macroscopic and microscopic ionizations in model compounds and in Z-Lys-CH₂-trypsin and Tos-Phe-CH₂-chymotrypsin inhibitor derivatives

R	R'	Solvent (w/w)*	Method used†	Microscopic pK _a values				Macroscopic pK _a values‡		Ratio of species (ii)/(iii)
				pK ₁	pK ₂	pK ₃	pK ₄	pK _I	pK _{II}	
CF ₃	H	100 % Water	Calc.	6.4	8.5	9.6	7.5	6.4	9.6	126
CF ₃	H	100 % Water	Exp.	6.3	8.2	9.8	7.9	6.3	9.8	79
CF ₃	H	50 % Dioxan	Exp.	5.9	9.7	10.8	7.0	5.9	10.8	6310
CF ₃	H	70 % Methanol	Exp.	5.9	9.6	10.8	7.1	5.9	10.8	5012
CF ₃	CH ₃	70 % Methanol	Exp.	5.7	8.9	10.2	7.0	5.7	10.2	1585
CF ₃	CH ₃	100 % Water	Calc.	6.3	8.5	9.6	7.4	6.3	9.6	158
Chymotrypsin		100 % Water	Calc.	6.7	10.7	11.9	7.9	6.7	11.9	10000
Trypsin		100 % Water	Calc.	6.7	10.7	11.9	7.9	6.7	11.9	10000
Chymotrypsin		100 % Water	Exp.	> 8.9	8.9	< pK ₄	> 11	8.9	> 11	≪ 1
Trypsin		100 % Water	Exp.	> 7.9	7.9	< pK ₄	> 11	7.9	> 11	≪ 1

* Co-solvent is water.

† Free-energy relationships (see the Materials and methods section) were used to calculate microscopic pK_a values in 100 % (w/w) water solutions. Experimental values were determined assuming that for compound (a) pK₁ = pK_I, pK₃ = pK_{II}, and that pK₂ = pK_a of compound (b) under the same experimental conditions (see the text for further details). pK₄ was determined using the relationship pK₄ = pK₃ + pK_I - pK₂.‡ These are the mean values of the appropriate pK_a values in Tables 1 and 2.

intact enzyme-inhibitor derivatives, whereas oxyanion formation is expected to produce larger positive titration shifts of 2.6–2.9 p.p.m. and 4.0–4.4 p.p.m. at the α - and β -carbon atoms respectively, irrespective of whether the imidazole group is neutral (pK₃; Scheme 2) or protonated and positively charged (pK₂; Scheme 2). Therefore the large positive titration shifts of 4.1–4.6 p.p.m. at the α -methylene and β -hemiketal carbon atoms in the intact trypsin- and chymotrypsin-chloromethane-inhibitor derivatives (Table 1) must primarily reflect oxyanion formation (pK₂ or pK₃; Scheme 2). If pK₁ = pK₂ (Scheme 2), then from our results with model hemiketals (Table 3) we predict that titration shifts of 0.5 p.p.m. and 2.7 p.p.m. would be observed at the α -methylene and β -hemiketal carbon atoms respectively. The observed α - and β -carbon titration shifts (4.1–4.6 p.p.m.; Table 1) in the intact enzyme-inhibitor derivatives are significantly larger than these predicted values showing that pK₁ $\neq$ pK₂ and that the observed titration shifts are due to oxyanion formation (pK₂ or pK₃; Scheme 2). Therefore, as only one titration shift is observed from pH 3 to 10.5 in the intact enzyme-inhibitor derivatives, the second macroscopic pK_a value must be either < 3 or > 10.5 and it will be due to the ionization of histidine-57 (pK₁ or pK₄; Scheme 2).

In the intact enzyme-chloromethane-inhibitor derivatives, N-3 of histidine-57 is alkylated, and deprotonation of the imidazolium cation of histidine-57 will result in the loss of the N-1 histidine-57-aspartic acid-102 hydrogen-bonded proton. This proton has

been directly observed by ¹H n.m.r. in the Tos-Phe-CH₂- δ -chymotrypsin and Z-Gly-Leu-Phe- δ -chymotrypsin derivatives and it was not lost from pH 4 to 9.5 (Robillard & Shulman, 1972, 1974a). Therefore histidine-57 is protonated up to pH 9.5 and the zwitterionic form of the tetrahedral adduct [species (iii); Scheme 2] predominated ($K_T \ll 1$; Scheme 2) over its neutral form [species (ii); Scheme 2] and so pK_I $\approx$ pK₂ and pK_{II} $\approx$ pK₄ (Scheme 2) with pK₄ > 9.5. As we have found no evidence for a negative α -methylene-carbon-atom titration shift in the intact Tos-[1-¹³C]-Phe-CH₂- δ -chymotrypsin derivative up to pH 10.5 (Fig. 3), we conclude that pK₄ > 11. Since $K_T \ll 1$, the concentration of the neutral tautomer [species (ii), Scheme 2] is negligible and the titration shift associated with pK_I (Scheme 2) is not detectable in the enzyme-inhibitor derivatives. But since $K_T = K_1/K_2$ and as $K_T \ll 1$, we conclude that pK_I $\gg$ pK₂. Therefore, in the enzyme-chloromethane-inhibitor derivatives, the pK_a of histidine-57 (pK₁; Scheme 2) is raised to a value greater than that of the oxyanion (pK₂; Scheme 3), even though the negatively charged oxyanion has not been formed.

The chemical shift of the N-1 hydrogen-bonded proton of the imidazolium cation of histidine-57 is decreased when negatively charged inhibitors bind to the serine proteinases (Robillard & Shulman, 1972, 1974b; Bachovchin *et al.*, 1988). Likewise the chemical shift of the hydrogen-bonded proton was decreased (–1 p.p.m.) on raising the pH of solutions of Tos-Phe-CH₂- δ -chymotrypsin and Z-Gly-Leu-Phe- δ -chymotrypsin (Robillard &

Fig. 4. pH titration of the α - and β -carbon atoms of compounds (a) and (b) in 100% (v/v) water

Acquisition parameters and sample conditions were as described in the Materials and methods section. ●, Chemical shifts (δ) of the α - and β -carbon atoms of compound (a). The continuous lines for the α - and β -carbon data were calculated by using eqn. (ii):

$$\delta_{\text{obs.}} = \frac{S_1 \cdot [\text{H}]^2 + S_2 \cdot K_1 \cdot [\text{H}] + S_3 \cdot K_1 \cdot K_{\text{II}}}{[\text{H}]^2 + K_1 \cdot [\text{H}] + K_1 \cdot K_{\text{II}}} \quad (\text{ii})$$

and the following fitted parameters: α -carbon: $pK_1 = 6.27 \pm 0.08$, $pK_{\text{II}} = 9.85 \pm 0.07$, $S_1 = 52.74 \pm 0.02$ p.p.m., $S_2 = 51.34 \pm 0.03$ p.p.m. and $S_3 = 53.74 \pm 0.02$ p.p.m.; β -carbon: $pK_1 = 6.26 \pm 0.06$, $pK_{\text{II}} = 9.83 \pm 0.05$, $S_1 = 92.27 \pm 0.01$ p.p.m., $S_2 = 92.95 \pm 0.01$ p.p.m. and $S_3 = 96.16 \pm 0.02$ p.p.m. ▲, Chemical shifts of the α - and β -carbon atoms of compound (b). The continuous lines for the α - and β -carbon data were calculated, using eqn. (i) as described in the legend to Fig. 3, by using the following fitted parameters: α -carbon: $pK_1 = 8.21 \pm 0.02$, $S_1 = 52.80 \pm 0.01$ p.p.m. and $S_2 = 55.35 \pm 0.01$ p.p.m.; β -carbon: $pK_1 = 8.23 \pm 0.02$, $S_1 = 92.03 \pm 0.01$ p.p.m. and $S_2 = 95.32 \pm 0.01$ p.p.m.

Shulman, 1972, 1974a). The pK_a of 8.4 determined from this titration shift at 16 °C is similar to that determined from the ^{13}C titration shift of the hemiketal carbon atom of the intact Tos-[2- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin derivative at 26 °C (Table 1), which we have assigned to oxyanion formation (pK_2 ; Scheme 2). Therefore we also assign the titration shift of the hydrogen-bonded proton to oxyanion formation.

Stabilization of zwitterionic tetrahedral intermediates in the intact Z-Lys-CH $_2$ -trypsin and Tos-Phe-CH $_2$ - δ -chymotrypsin derivatives

In the intact trypsin and δ -chymotrypsin-chloromethane-inhibitor derivatives, the pK_2 values of the oxyanion are lowered

Table 3. Titration constants obtained by ^{13}C n.m.r. studies on compounds (a) and (b)

The errors associated with the chemical shifts and pK_s values are less than 0.1 p.p.m. and 0.1 pH unit respectively.

Compound†	Species	Solvent (w/w)§	α -Carbon atom				β -Carbon atom			
			pH-independent* chemical shifts (p.p.m.)		Titration shift† (p.p.m.)		pH-independent* chemical shifts (p.p.m.)		Titration shift† (p.p.m.)	
			δ_1	δ_2	δ_1	$\delta_2 - \delta_1$	δ_1	δ_2	$\delta_2 - \delta_1$	$\delta_3 - \delta_2$
(a)	Hydrate	100% Water	52.74	51.34	-1.40	2.40	92.27	92.95	0.68	3.21
(a)	Hydrate	50% Dioxan	52.85	51.08	-1.77	2.68	92.17	92.65	0.48	2.98
(a)	Hydrate	70% Methanol	53.00	51.47	-1.53	2.61	92.31	93.06	0.75	3.19
(a)	Hemiketal	70% Methanol	52.01	50.11	-1.90	2.59	94.68	95.57	0.89	3.97
(b)	Hydrate	100% Water	52.80	55.34	2.54	2.54	92.03	95.32	3.29	8.23
(b)	Hydrate	50% Dioxan	53.12	55.71	2.59	2.59	92.18	95.38	3.20	9.68
(b)	Hydrate	70% Methanol	53.44	55.97	2.53	2.53	92.32	95.55	3.23	9.62
(b)	Hemiketal	70% Methanol	52.49	55.36	2.87	2.87	94.45	98.83	4.38	8.82
(a)	Hydrate	100% Water	53.74	53.74	-	-	96.16	96.16	-	-
(a)	Hydrate	50% Dioxan	53.76	53.76	-	-	95.63	95.63	-	-
(a)	Hydrate	70% Methanol	54.08	54.08	-	-	96.25	96.25	-	-
(a)	Hemiketal	70% Methanol	52.70	52.70	-	-	99.54	99.54	-	-
(b)	Hydrate	100% Water	52.80	55.34	2.54	2.54	92.03	95.32	3.29	8.23
(b)	Hydrate	50% Dioxan	53.12	55.71	2.59	2.59	92.18	95.38	3.20	9.68
(b)	Hydrate	70% Methanol	53.44	55.97	2.53	2.53	92.32	95.55	3.23	9.62
(b)	Hemiketal	70% Methanol	52.49	55.36	2.87	2.87	94.45	98.83	4.38	8.82

* δ_1 is the chemical shift of the fully protonated compound; δ_2 is the chemical shift after the first macroscopic ionization is complete (pK_1); δ_3 is the chemical shift after the second macroscopic ionization is complete (pK_{II}).

† $\delta_2 - \delta_1$ is the titration shift associated with pK_1 ; $\delta_3 - \delta_2$ is the titration shift associated with pK_{II} .

‡ The structures of compounds (a) and (b) are given in Fig. 1.

§ The co-solvent is in all cases water.

2.8 and 1.8 pK_a units respectively, relative to their values in fully aqueous media (Scheme 2). Also, pK_1 of the imidazolium cation is raised > 1.2 and > 2.2 pK_a units in the trypsin- and δ -chymotrypsin-chloromethane-inhibitor derivatives respectively relative to their values in water (Scheme 2). Therefore, in both of the enzyme-inhibitor derivatives, the net perturbation of pK_1 and pK_2 is at least 4 pK_a units. This results in both enzymes preferentially stabilizing the zwitterionic form of the tetrahedral adduct [species (iii); Scheme 2], as opposed to its neutral form [species (ii); Scheme 2], which we predict would predominate in water. Therefore the concentration of the zwitterionic tetrahedral adduct in the enzyme-inhibitor derivative ($K_T \ll 1$; Scheme 2) is at least 10000-fold greater than its value in water ($K_T = 10000$; Scheme 2) and so both enzymes must contribute at least $22.8 \text{ kJ} \cdot \text{mol}^{-1}$ to the stabilization of the zwitterionic tetrahedral adduct at 25°C .

In order for the zwitterionic tetrahedral adduct to predominate over its neutral form, the tautomeric ratio ($K_T = K_1/K_2$; Scheme 2) must be less than 1, and so pK_1 must be greater than pK_2 (Scheme 2). Therefore, in the trypsin- and δ -chymotrypsin-inhibitor derivatives, pK_1 of the imidazolium cation must be > 7.9 and > 8.9 respectively (Scheme 2). In earlier studies (Malthouse *et al.*, 1985; Finucane *et al.*, 1989) it was suggested that pK_2 of the oxyanion was lowered as a result of its interaction with the imidazolium ion of histidine-57. If the effective dielectric constant between these groups is 13.0 in the Z-Lys-CH₂-trypsin derivative and 17.3 in the Tos-Phe-CH₂- δ -chymotrypsin derivative, then pK_2 of the oxyanion will be lowered 4 and 3 pK_a units respectively, relative to pK_3 of the oxyanion, whereas the pK_4 values of the imidazolium cation should increase by equal amounts relative to pK_1 of the imidazolium cation (Scheme 2). But when the dielectric constant of the solvent containing compound (a) was decreased from 78.5 to 35–45, both pK_2 and pK_3 of the oxyanion increased, and this increase in pK_2 offset the decrease in the oxyanion pK_a due to its interaction with the imidazolium cation (Scheme 2). Therefore, if the dielectric constant was decreased further to values of 13 or 17.3, pK_2 would be expected to increase relative to the value of pK_3 in water (Scheme 2). It has been argued that the specific solvation of charged groups in proteins can stabilize these charges as well as in water, and so the local effective dielectric constant in the immediate vicinity of the group can be similar to that of water, and the pK_a values will be similar to their values in water (Warshel, 1979; Warshel & Russell, 1984; Warshel *et al.*, 1984; Russell & Warshel, 1985). We suggest that the primary role of hydrogen-bonding to the oxyanion in the oxyanion hole may be to maintain pK_3 of the oxyanion at a value similar to its value in water and so allow the electrostatic interaction with the imidazolium cation of histidine-57 to reduce pK_2 to the experimentally observed pK_a values in the intact enzyme-inhibitor derivatives (Scheme 2).

In contrast with the oxyanion pK_a values, pK_1 of the imidazolium cation is expected to be insensitive to the dielectric constant (Edsall & Wyman, 1958), and our results with compound (a) show that pK_1 decreases as the dielectric constant of the solvent is decreased (Scheme 2). Therefore this simple model, which only considers a charge–charge interaction between the oxyanion and the imidazolium cation, does not explain how pK_1 of the imidazolium cation is raised from its value of 6.7 in water to a value $> pK_2$ in both enzyme-inhibitor derivatives. But with a model compound containing a phenolic hydroxy group (OH), an imidazole group (ImH) and a carboxylate (CO₂H) group where $pK_a^{\text{OH}} > pK_a^{\text{ImH}} > pK_a^{\text{CO}_2\text{H}}$, it was found that the pK_a of the imidazolium cation increased as the dielectric constant of the solvent decreased (Rogers & Bruice, 1974). This suggests that the carboxylate anion of aspartate-102 in the trypsin- and

chymotrypsin-inhibitor derivatives may play an important role in raising pK_1 to a value $> pK_2$ in these inhibitor derivatives. But when aspartate-102 of trypsin is replaced by an asparagine residue, the pK_a of histidine-57 in the free enzyme is only decreased by 1.5 pK_a units from a value of 7 to 5.5 (Sprang *et al.*, 1987). This implies that in the free enzyme the effective dielectric constant between the charges of the imidazolium cation and the carboxylate anion is 44 assuming a charge separation of 3.7 Å. This value of the effective dielectric constant is typical of that expected for charge–charge interactions between surface groups on a protein (Warshel & Russell, 1984; Warshel *et al.*, 1989). It has been suggested (Warshel *et al.*, 1989) that this electrostatic interaction is partly offset by the free energy ($8.37 \text{ kJ} \cdot \text{mol}^{-1}$) required to prevent the 180° inversion of the imidazole ring of histidine-57 (Sprang *et al.*, 1987). Therefore, if in the intact Z-Lys-CH₂-trypsin and Tos-Phe-CH₂- δ -chymotrypsin derivatives the imidazole ring of histidine-57 is 'locked in position', then the effect of the full electrostatic interaction will be manifested as an increase in pK_1 , and its pK_a will be raised to a value of approx. 8.5 as the effective dielectric constant will be 22. Secondly, Warshel *et al.* (1989) have suggested that, in the free enzyme the charge–charge interaction between the imidazolium cation of histidine-57 and the carboxylate anion of aspartate-102 is largely compensated for by the solvation effects at the solvent-exposed N-3 of the imidazole ring. If this compensating solvation effect is lost as a result of alkylation of N-3 of the imidazole ring of histidine-57 in the enzyme-chloromethane-inhibitor derivatives, then this could also increase pK_1 . Thirdly, Warshel *et al.* (1989) have suggested that the exclusion of water from the active site on substrate binding could decrease the effective dielectric constant between the imidazolium ion and carboxylate anion. If a similar effect occurs in the enzyme-chloromethane-inhibitor derivatives, this could also raise pK_1 . Therefore any one, two, or all three, of these factors could contribute to raising pK_1 in the intact Z-Lys-CH₂-trypsin and Tos-Phe-CH₂- δ -chymotrypsin derivatives. If we assume that, in the intact Tos-Phe-CH₂- δ -chymotrypsin derivative, there is a uniform effective dielectric constant of 8 at the active site and that pK_1 and pK_3 (Scheme 2) in the presence of a neutral carboxylate group have pK_a values of 6.7 and 11.9 respectively, then the values of pK_1 , pK_2 , pK_3 and pK_4 in the presence of the carboxylate anion of aspartate-102 can be estimated (see the Materials and methods section). pK_1 will be raised from 6.7 to 14.1, and pK_3 from 11.9 to 15.4, owing to their interaction with the carboxylate anion. pK_4 will be 20.6, owing to its interaction with the carboxylate anion ($\Delta pK_a = +7.4$) and the oxyanion ($\Delta pK_a = +6.5$). pK_2 will be lower than pK_3 , owing to its interaction with the imidazolium cation ($\Delta pK_a = -6.5$) and so $pK_2 = 8.9$. Likewise, if the effective dielectric constant at the active site of Z-Lys-CH₂-trypsin derivative is 6, then $pK_1 = 16.6$, $pK_2 = 7.9$, $pK_3 = 16.5$, $pK_4 = 25.2$. These are not unique solutions for these pK_a values; e.g., if in the Tos-Phe-CH₂- δ -chymotrypsin derivative the effective dielectric constant between the oxyanion and the carboxylate anion, the oxyanion and the imidazolium cation, the imidazolium and the carboxylate anion are 22, 12.2 and 18 respectively, then $pK_1 = 10.0$, $pK_2 = 8.9$, $pK_3 = 13.2$ and $pK_4 = 14.3$. If the effective dielectric constant between the oxyanion and the imidazolium cation is reduced from 12.2 to 9.9 in the Z-Lys-CH₂-trypsin derivative, then $pK_1 = 10.0$, $pK_2 = 7.9$, $pK_3 = 13.2$ and $pK_4 = 15.3$. These calculations illustrate that the lowering of the oxyanion pK_a in the trypsin- and δ -chymotrypsin-chloromethane derivatives and the preferential stabilization of the zwitterionic tetrahedral adduct ($pK_1 > pK_2$, $K_T < 1$, Scheme 2) can be explained in terms of electrostatic charge–charge interactions, provided specific solvation effects offset increases in pK_a values due to decreases in the dielectric constant.

When chymotrypsin is inhibited by a trifluoromethane inhibitor which does not alkylate N-3 of the imidazole ring of histidine-57, pK_a (Scheme 2) of the oxyanion of the tetrahedral adduct is lowered at least 5 pK_a units to a value of < 4 , whereas pK_a (Scheme 2) of the imidazolium cation of histidine-57 is raised to a value > 9.5 in the enzyme-inhibitor complex (Liang & Abeles, 1987; Brady *et al.*, 1989). Therefore, since the zwitterionic tetrahedral adduct predominates, pK_1 of histidine-57 must lie in the range 4–9.5, but we cannot deduce whether $pK_1 > 7$ in this enzyme-inhibitor complex. Recently, Ortiz *et al.* (1991) have shown that, when an aldehyde inhibitor binds to chymotrypsin, pK_a of the oxyanion of the tetrahedral adduct is lowered 8.6 pH units relative to the value of 13.3 expected for the oxyanion of a hemiacetal in water (Fastrez, 1983). A small titration shift was also detected for the C-2 proton of the imidazole ring of histidine-57. If, as Ortiz *et al.* (1991) suggest, this titration shift may not represent ionization of histidine-57, then the zwitterionic tetrahedral adduct predominates with $pK_a > 8.5$ and pK_1 lying in the range 4.7–8.5. Thus, in this case, we cannot deduce whether $pK_1 > 7$ in this enzyme-inhibitor complex. However, if the titration shift of the C-2 proton does reflect ionization of histidine-57, then we estimate that approx. 20% of histidine-57 has a $pK_a = 5.4$ (pK_1 ; Scheme 2) and the remaining 80% has a $pK_a > 8.5$ (pK_1 ; Scheme 2) in this enzyme-inhibitor complex. If this interpretation of the experimental data is correct, then the lack of an increase in pK_1 of histidine-57 may reflect the inability of the aldehyde inhibitor to fix the imidazole ring of histidine-57 in the correct position for catalysis and to exclude water from the appropriate region of the active site. However, it is possible that, with larger polypeptide substrates and inhibitors which bind in both S_1 and S_1' subsites, pK_1 of histidine-57 will be > 7 both before and after tetrahedral-intermediate formation.

It has been argued (Warshel *et al.*, 1989) that the folding energy of the serine proteinases can be used to align the hydrogen bonds to the oxyanion in the oxyanion hole such that they act as pre-aligned dipoles which can stabilize the oxyanion to a greater extent than in water because solvent reorganization is avoided (Warshel, 1978; Warshel *et al.*, 1989). But when one of the hydrogen-bond donors in the oxyanion hole of subtilisin is removed, it is estimated that oxyanion formation is destabilized by 9–20 $\text{kJ} \cdot \text{mol}^{-1}$ (Bryan *et al.*, 1986; Wells *et al.*, 1986). This destabilization is very similar to that expected when a single 'normal hydrogen bond' (i.e. not involving two or more pre-aligned dipoles) between a neutral donor and a charged acceptor is lost (Fersht *et al.*, 1985; Carter & Wells, 1990). Therefore, although hydrogen-bonding to the oxyanion in the oxyanion hole most probably plays an important role in maintaining the oxyanion pK_a at a value similar to that expected in a fully aqueous medium, there is no experimental evidence that it plays a significant role in reducing the oxyanion pK_a to a value less than that expected in an aqueous environment.

Stabilization of tetrahedral intermediates during catalysis by serine proteinases

When a zwitterionic tetrahedral intermediate is fully formed during catalysis with a peptide substrate, the nitrogen atom of the peptide bond is expected to have a $pK_a \approx 10$ in an aqueous environment (Satterthwait & Jencks, 1974; Komiyama & Bender, 1979). But for optimal general-acid or general-base catalysis the difference between the pK_a values of the proton donors and acceptors should be minimized. Therefore the pK_a of the imidazolium cation of histidine-57 should be raised to a value of ≈ 10 if it is to act as an efficient general acid catalyst for the breakdown of the tetrahedral intermediate. This assumes that the pK_a of the nitrogen atom of the leaving group is not perturbed in the enzyme-bound tetrahedral intermediate. Since it

Scheme 3. Kinetic scheme for the pH dependence of catalysis when ES and ESH do not exchange protons with the solvent

For further details, see the text.

is the oxyanion and not its conjugate acid that is expected to be the catalytically relevant species (Malthouse *et al.*, 1985), then for optimal catalytic efficiency the serine proteinases should stabilize a zwitterionic tetrahedral intermediate during catalysis, analogously to the zwitterionic tetrahedral adduct [species (iii); Scheme 2] present in the chloromethane-inhibitor derivatives. For this zwitterionic tetrahedral intermediate, pK_1 of the imidazolium cation of histidine-57 must be $> pK_a$ of the oxyanion, and pK_a of the imidazolium cation should be ≈ 10 , with $pK_a > pK_1 > pK_2$ (Scheme 3). pK_a may be > 10 if the pK_a of the nitrogen atom of the leaving group is > 10 or if the general acid catalysis of leaving-group expulsion is not optimized.

Although there is not expected to be significant proton transfer from the hydroxy group of serine-195 ($pK_a = 15$ –16) to the imidazole group of histidine-57 ($pK_a \approx 7$) in the free enzyme (Blow, 1976; Bachovchin, 1986), it is generally accepted that the nucleophilicity of the hydroxy group of serine-195 is enhanced by the imidazole group of histidine-57 acting as a general base catalyst (Fersht, 1985; Bachovchin, 1986; Fink, 1987). Therefore if substrate binding raises the pK_a of the imidazolium cation before tetrahedral-intermediate formation, this should enhance the nucleophilicity of the hydroxy group of serine-195 by increasing the efficiency of the general-base-catalysed removal of the proton of the hydroxy group of serine-195 by the imidazole group of histidine-57. But if this occurs, we would expect the pK_a values determined from the pH-dependence of k_{cat} values to exceed those determined from the pH-dependence of k_{cat}/K_m values, which are thought to reflect the ionization of the imidazolium cation ($pK_a \approx 7$) of histidine-57 (Fersht, 1985). However, this is not observed, and usually the pK_a values determined from the pH-dependent changes in k_{cat} values are $< pK_a$ values determined from pH-dependent changes in the values of k_{cat}/K_m for both chymotrypsin (Fersht, 1985) and trypsin (Spomer & Wotton, 1971)-catalysed reactions involving amide, peptide or anilide substrates. This would appear to suggest that the pK_a of the imidazolium cation of histidine-57 cannot be raised in the enzyme-substrate complex before tetrahedral-intermediate formation. But if, after substrate binding, N-1 and N-3 of the imidazole or imidazolium group of histidine-57 are unable to accept or donate protons to the solvent at a significant rate compared with k_{cat} , then the increased pK_a of the imidazolium cation will not be manifested in the pH-dependence of k_{cat} . A kinetic scheme depicting this situation is depicted in Scheme 3. In this Scheme, E and ES are the free enzyme and enzyme-substrate complex respectively when the imidazole ring of histidine-57 is neutral. EH and EHS are the free enzyme and the enzyme-substrate complex respectively when the imidazolium cation of histidine-57 is present. K_s and K_s' are the dissociation constants for the enzyme-substrate complexes of ES and EHS respectively. K_a is the dissociation constant of the imidazolium cation of histidine-57 in the free enzyme. For amide, peptide and anilide substrates it is assumed that the rate of acylation (k_2) is rate-limiting and that k_2 is slow relative to the

pH-equilibration steps of Scheme 3. The catalytic parameters (k_{cat} , K_{m} and $k_{\text{cat}}/K_{\text{m}}$) defining catalytic turnover for Scheme 3 are given below:

$$k_{\text{cat.}} = \frac{k_2}{1 + [\text{H}]/K_{\text{obs.}}}$$

$$K_{\text{m}} = \frac{K_{\text{s}}(1 + [\text{H}]/K_{\text{a}})}{1 + [\text{H}]/K_{\text{obs.}}}$$

$$\frac{k_{\text{cat.}}}{K_{\text{m}}} = \frac{k_2/K_{\text{s}}}{1 + [\text{H}]/K_{\text{a}}}$$

$$K_{\text{obs.}} = K_{\text{a}} \cdot K_{\text{s}}'/K_{\text{s}}$$

Therefore, although the pH-dependent changes in $k_{\text{cat.}}/K_{\text{m}}$ will reflect the dissociation constant (K_{a}) of the imidazolium cation in the free enzyme, the pH-dependence of $k_{\text{cat.}}$ will reflect $K_{\text{obs.}}$ ($K_{\text{obs.}} = K_{\text{a}} \cdot K_{\text{s}}'/K_{\text{s}}$). If the substrate binds less tightly to EH than to E ($K_{\text{s}}' > K_{\text{s}}$), then the $\text{p}K_{\text{a}}$ determined from the pH-dependence of $k_{\text{cat.}}$ will be less than that determined from the pH-dependent changes in $k_{\text{cat.}}/K_{\text{m}}$. Therefore, provided that the ES and ESH derivatives do not exchange protons with the solvent at a significant rate relative to k_2 , then this will explain why $\text{p}K_{\text{a}}$ values determined from the pH-dependence of $k_{\text{cat.}}$ are $< \text{p}K_{\text{a}}$ values determined from the pH-dependence of $k_{\text{cat.}}/K_{\text{m}}$. The discrepancy between the $\text{p}K_{\text{a}}$ values determined from the pH-dependent changes in the values of $k_{\text{cat.}}$ and $k_{\text{cat.}}/K_{\text{m}}$ for anilide substrates has been explained by a similar mechanism in which chymotrypsin can bind substrates in a productive mode and a non-productive mode (Fastrez & Fersht, 1973). Such results attributed to a non-productive mode could also be explained by Scheme 3 if this situation applies. However, when formyl-L-phenylalanine formylhydrazide or formyl-L-phenylalanine semicarbazide bind to chymotrypsin, the number of protons released suggests that the $\text{p}K_{\text{a}}$ of the imidazolium cation of histidine-57 is lowered to a value of 6.2–6.4, as was suggested by the pH-dependence of $k_{\text{cat.}}$ values (Fersht & Renard, 1974). This apparent decrease in the $\text{p}K_{\text{a}}$ of histidine-57 may be due to the fact that with these substrates a nitrogen atom replaces the α -carbon present in a normal substrate (Fersht & Renard, 1974) or it could reflect the decrease in the $\text{p}K_{\text{a}}$ of some other ionizing group on substrate binding.

General summary and conclusions

Our results with the trypsin and δ -chymotrypsin-chloromethane-inhibitor derivatives show that, in these derivatives, the zwitterionic tetrahedral adduct is stabilized by lowering $\text{p}K_2$ of the oxyanion and raising $\text{p}K_1$ of the imidazolium cation (Scheme 2). Provided that the localized solvation of the oxyanion in the oxyanion hole prevents an increase in $\text{p}K_3$ of the oxyanion when the effective dielectric constant between the charges in the active site is less than that of water, then the decrease in $\text{p}K_2$ of the oxyanion can be explained by its electrostatic interaction with the imidazolium cation of histidine-57. The increase in $\text{p}K_1$ of the imidazolium cation of histidine-57 can be accounted for by an increased electrostatic interaction between the carboxylate anion of aspartate-102 and the imidazolium cation of histidine-57, resulting from a decrease in the effective dielectric constant between these groups when N-3 of the imidazole ring of histidine-57 is alkylated by chloromethane inhibitors. The possibility that $\text{p}K_2$ of the oxyanion is lowered by hydrogen bonds in the oxyanion hole acting as pre-aligned dipoles cannot be dismissed on the basis of our results. We conclude that the decrease in $\text{p}K_2$ of the oxyanion could result from either hydrogen-bonding in the oxyanion hole, or as a result of its interaction with the

imidazolium cation of histidine-57, or a combination of both these factors. But we suggest that the increase in $\text{p}K_1$ of the imidazolium cation must reflect an increase in the magnitude of its electrostatic interaction with the carboxylate anion of aspartate-102 due to the decrease in the effective dielectric constant between these groups when N-3 of the imidazole ring of histidine-57 is alkylated.

The fact that the concentration of the zwitterionic tetrahedral adduct [species (iii); Scheme 2] is at least 10000-fold greater in the trypsin and δ -chymotrypsin-chloromethane-inhibitor derivatives than in water (Scheme 2) is consistent with the suggestion that these enzymes have evolved to stabilize preferentially the zwitterionic form of tetrahedral intermediates during catalysis and that this stabilization is an important factor in ensuring the catalytic efficiency of these enzymes. We expect the stabilization of zwitterionic tetrahedral intermediates formed during catalysis to occur by mechanisms similar to those we have proposed for the stabilization of zwitterionic tetrahedral adducts in the trypsin and δ -chymotrypsin-chloromethane-inhibitor derivatives. But $\text{p}K_1$ of the imidazolium cation of histidine-57 may not be increased when tetrahedral intermediates are formed during catalysis because N-3 of the imidazole ring of histidine-57 is not alkylated as in the enzyme-chloromethane-inhibitor derivatives. However, if when the tetrahedral intermediate is formed the substrate fixes the imidazole ring of histidine-57 in the correct position for catalysis, and if it excludes water from the active site, then these factors may decrease the effective dielectric constant between the carboxylate anion of aspartate-102 and the imidazolium cation of histidine-57. This would increase the electrostatic interaction between these groups and raise $\text{p}K_1$ of the imidazolium cation. If the $\text{p}K_{\text{a}}$ of the imidazolium cation of histidine-57 is increased by the same process before tetrahedral-intermediate formation, then this could explain the enhanced nucleophilicity of the hydroxy group of serine-195. The fact that the $\text{p}K_{\text{a}}$ values determined from the pH-dependence of $k_{\text{cat.}}$ values are generally less than, or equal to, $\text{p}K_{\text{a}}$ values determined from the pH-dependence of $k_{\text{cat.}}/K_{\text{m}}$ values can be explained by Scheme 3, provided the substrate binds equally or less well to the enzyme when the positively charged imidazolium cation of histidine-57 is present than when its neutral conjugate base is present.

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