Determination of the ionization state of the active-site histidine in a subtilisin–(chloromethane inhibitor) derivative by ^{13}C -NMR

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Subtilisin BPN' has been alkylated using benzyloxycarbonyl-glycylglycyl[1- ^{13}C]phenylalanylchloromethane. Using difference ^{13}C -NMR spectroscopy a single signal due to the ^{13}C -enriched α -methylene carbon of the subtilisin–(chloromethane inhibitor) derivative was detected. No evidence for the denaturation/autolysis of this derivative was obtained from pH 3.5 to 11.5. However, incubating at pH 12.75 or heating in the presence of SDS at pH 6.9 did denature this derivative. The negative titration shift of the α -methylene carbon of the denatured derivatives confirmed that the inhibitor had alkylated N-3 of the imidazole ring of the active-site histidine. The positive titration shift of 3.96 p.p.m. and the pK_a of 7.04 obtained from studying the

native subtilisin–(chloromethane inhibitor) derivative are assigned to oxyanion formation. We conclude that the pK_a of the alkylated histidine residue in the native subtilisin–(chloromethane inhibitor) derivative must be > 12 and that subtilisin preferentially stabilizes the zwitterionic tetrahedral adduct consisting of the oxyanion and the imidazolium ion of the active-site histidine residue. We show that even before the oxyanion is formed the pK_a of the active-site histidine must be much greater than that of the oxyanion in the zwitterionic tetrahedral adduct. We discuss the significance of our results for the catalytic mechanism of the serine proteinases.

INTRODUCTION

N-3 of the imidazole ring of the active-site histidine residue of trypsin [1,2], chymotrypsin [3,4] and subtilisin [5–7] can be alkylated by specific substrate-derived chloromethane inhibitors. ^{13}C -NMR has been used to show that in trypsin– [8–10] chymotrypsin– [11,12] and subtilisin–(chloromethane inhibitor) derivatives [13] the hydroxy group of the active-site serine adds to the ketone carbon of the inhibitor to form a tetrahedral adduct analogous to the tetrahedral intermediate thought to be formed during catalysis by the serine proteinases. By determining the pH-dependent changes in the chemical shift of the hemiketal carbon of these tetrahedral adducts it has been concluded that the pK_a of the oxyanion is 7.9 and 8.9 in the trypsin– and δ -chymotrypsin–inhibitor derivatives respectively [9,11]. Using ^{13}C -NMR it has been shown that the titration shift of the α -methylene carbon of both the trypsin– and δ -chymotrypsin–inhibitor derivatives can be used to determine whether either oxyanion formation or deprotonation of the alkylated imidazolium ion is occurring [12,14]. In model compounds of chloromethane inhibitor derivatives the ionization of the oxyanion will cause an approx. 4–5 p.p.m. increase in the chemical shift of the inhibitor α -methylene carbon [12,14]. However, deprotonation of the alkylated imidazolium ion will produce an approx. 1.5–2.0 p.p.m. decrease in chemical shift [12,14]. Therefore the large positive titration shift which was observed with the α -methylene carbon of both the intact trypsin– and the intact δ -chymotrypsin–inhibitor derivatives was assigned to oxyanion formation [12,14]. There was no evidence for a negative titration up to pH 10.5, and so it was concluded that the pK_a of the imidazolium ion of the active-site histidine was > 11 in both the intact trypsin– and the intact chymotrypsin–(chloromethane inhibitor) derivatives [12,14].

When subtilisin was inhibited with benzyloxycarbonyl-glycylglycyl[2- ^{13}C]phenylalanylchloromethane (Z-Gly-Gly-[2-

^{13}C]Phe-CH₂Cl), the magnitude of the titration shift of the hemiketal carbon was similar to that observed in trypsin– and chymotrypsin–(chloromethane inhibitor) derivatives [13]. However, the pK_a determined from this titration shift was 6.9, which is essentially the same as the pK_a of the active-site histidine in unmodified subtilisin [15–19]. This pK_a is much lower than that determined from hemiketal titration shifts in chymotrypsin– and trypsin–(chloromethane inhibitor) derivatives, which led to the suggestion that oxyanion stabilization is more efficient in the subtilisin–chloromethane derivative. It is therefore important that we determine whether this pK_a is due to formation of the oxyanion or to the ionization of the imidazolium ion of the active-site histidine residue.

It has been suggested that both chymotrypsin and trypsin may have evolved to stabilize zwitterionic tetrahedral intermediates and that this stabilization is an important factor in ensuring the catalytic efficiency of these enzymes [9,11,12]. The positive and negative charges of this zwitterionic tetrahedral intermediate would be provided by the oxyanion and the imidazolium cation of the active-site histidine residue. The trypsin– and chymotrypsin–(chloromethane inhibitor) derivatives have been shown to stabilize zwitterionic tetrahedral adducts and so form good models for catalytic tetrahedral intermediates. However, while subtilisin (a bacterial serine proteinase) has a three-dimensional structure quite different from that of the mammalian serine proteinases, it also has a catalytic triad and an oxyanion hole, which suggests that convergent evolution has produced two different serine proteinases with similar catalytic mechanisms [20,21]. It is therefore important that we determine whether subtilisin, like trypsin and chymotrypsin, also preferentially stabilizes zwitterionic tetrahedral adducts. However, for such a zwitterionic tetrahedral adduct to be stabilized, the pK_a of the active-site histidine in the presence of the conjugate acid of the oxyanion must be greater than the pK_a of the oxyanion in the presence of the imidazolium ion of the active-site histidine [12].

Abbreviations used: Z, benzyloxycarbonyl; Tos, tosyl.

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Therefore it is important that we determine whether the pK_a of the imidazolium ion of the active-site histidine is greater than that of the oxyanion in subtilisin–(chloromethane inhibitor) derivatives.

In the present work we have synthesized Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl and used it to inhibit subtilisin. We have determined the titration shift and pK_a value which determine the chemical shift of the α -methylene carbon in the intact and denatured inhibitor derivatives. This allows us to determine the following: (1) whether the inhibitor does alkylate the active-site histidine residue, (2) whether the titration shifts observed with the intact and denatured inhibitor derivatives are due to deprotonation of the active-site histidine or formation of the oxyanion, and (3) if the pK_a of the imidazolium ion of the active-site histidine is greater than that of the oxyanion.

MATERIALS AND METHODS

The materials and methods used were as described in our earlier study [13].

Synthesis of Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl

This was prepared as described previously for the synthesis of Z-Gly-Gly-Phe-CH₂Cl [13], except that *N*-[^{13}C]methyl-*N*-nitrosotoluene-*p*-sulphonamide was used to generate the diazomethane used in the synthesis of Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl.

The ^{13}C -NMR spectrum of Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl in [$^2\text{H}_6$]DMSO gave a triplet ($J_{\text{CD}} = 150$ Hz) centred at 48.1 p.p.m. in the absence of proton decoupling.

Alkylation of subtilisin by Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl

Samples of subtilisin were alkylated by Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl using the same methods used to alkylate chymotrypsin by Z-Gly-Gly-[2- ^{13}C]Phe-CH₂Cl [13].

Preparation of denatured Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin, Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin and Z-Gly-Gly-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin

Samples of alkali-denatured/autolysed Z-Gly-Gly-Phe-CH₂-subtilisin were prepared by raising the pH of a 2–3 mM solution of the protein containing 50 mM glycine and 50 mM glycyglycine to pH 12.75 and incubating at 20 °C for 4 h before lowering the pH to 7.0.

Samples of SDS-denatured Z-Gly-Gly-Phe-CH₂-subtilisin and Z-Gly-Gly-Phe-CH₂- δ -chymotrypsin were prepared by adding 5% (w/w) SDS to a 2–3 mM solution of the protein containing 50 mM glycine and 50 mM glycyglycine. This was then heated at 70 °C for 3 min.

pH titrations and analysis of pH-dependent changes in the chemical shift

These were carried out as described by Malthouse et al. [9], except that samples were titrated with 0.8 M KOH or 0.8 M HCl containing 20% (v/v) $^2\text{H}_2\text{O}$.

NMR spectroscopy

NMR spectra were recorded at 1.88 T with a Bruker WP80 wide-bore spectrometer operating at 20.115 MHz for ^{13}C nuclei. For Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin, alkali-denatured/autolysed Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin and SDS-denatured

Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin, the pulse sequence of Belton et al. [22] was used to minimize acoustic ringing. Spectral conditions were: 2048 data points per spectrum; spectral width of 220 p.p.m.; acquisition time of 0.05 s; 1 W broad-band decoupling. All spectra were zero-filled to 16384 data points. Exponential weighting factors of 40 Hz and 20 Hz were used when processing the free induction decays of the intact and denatured inhibitor derivatives respectively.

The samples and the conditions used for obtaining spectra of the samples of alkali-denatured/autolysed Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin, SDS-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin and SDS-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin were as described previously [13].

RESULTS AND DISCUSSION

Alkylation of subtilisin with Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl

When subtilisin was alkylated with Z-Gly-Gly-[1- ^{13}C]Phe-CH₂Cl at pH 7.07 (Figure 1a), one new signal was observed at 58.0 p.p.m. which was not present in the spectrum of the subtilisin inhibited by Z-Gly-Gly-Phe-CH₂Cl which was not ^{13}C -enriched (Figure 1b). The difference spectrum (Figure 1c) confirmed that only one new signal was observed. The chemical shift of this signal increased from 56.10 p.p.m. to 60.06 p.p.m. with increasing pH according to a pK_a of 7.04 (curve A in Figure 2).

Denaturation of enzyme–chloromethane derivatives

In our earlier studies [8,9,11] on the 2- ^{13}C -enriched trypsin- and 2- ^{13}C -enriched chymotrypsin–(chloromethane inhibitor) derivatives we have shown that in the intact derivative the ^{13}C -enriched carbon is sp^3 hybridized (structures A and B in Scheme 1) with a chemical shift of ~ 100 p.p.m. (Table 1). On alkali denaturation the ^{13}C -enriched carbon had a chemical shift of ~ 200 p.p.m. (Table 1), showing that it is sp^2 hybridized (structures C and D in Scheme 1). However, in an earlier study of Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin we obtained no evidence for the formation of a denatured species even after incubating the alkylated subtilisin for 8.8 h at pH 10.11 [13]. In the present work we show that the Z-Gly-Gly-Phe-CH₂-subtilisin derivative can be denatured by alkaline denaturation/autolysis at pH 12.75 or by heating in the presence of 5% (w/w) SDS.

Alkaline denaturation/autolysis caused by incubation of Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin at pH 12.75 for 4 h resulted in loss of the signal at 101.62 p.p.m. (Figure 1d) due to the sp^3 hybridized hemiketal carbon of the intact Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin derivative (structures A and B in Scheme 1), and in the appearance of a new signal at 205.10 p.p.m. (Figure 1e). The chemical shift of this signal increased from 202.49 p.p.m. to 205.40 p.p.m. with increasing pH according to a pK_a of 5.66 (curve B in Figure 2). The pK_a and titration shift of 2.91 p.p.m. of this signal are similar to those observed when Z-[2- ^{13}C]Lys-CH₂-trypsin, Z-Gly-Gly-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin, Z-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin and Tos-[2- ^{13}C]Phe-CH₂- α -chymotrypsin (Tos is tosyl) are denatured by alkaline denaturation/autolysis (Table 1), and we assign it in the same way to the sp^2 hybridized carbon of the denatured species (structures C and D in Scheme 1). Alkali-denatured samples of Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin (Figure 1f) and Z-Gly-Gly-Phe-CH₂-subtilisin (Figure 1g) were prepared at pH 6.89 and the difference spectrum (Figure 1h) contained a single signal at 54.55 p.p.m. due to the ^{13}C -enriched α -methylene carbon of the denatured Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin derivative. The chemical shift of the ^{13}C -enriched α -methylene carbon of the alkali-denatured Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin derivative decreased as the pH in-

Figure 1 ^{13}C -NMR spectra of native and denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin and Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin

Acquisition parameters were as described in the Materials and methods section. The numbers of transients recorded per spectrum (the exponential weighting factors used are in parentheses) were: 614 400 (40 Hz) for spectra (a) and (b); 24 576 (5 Hz) for spectra (d) and (e); 313 123 (20 Hz) for spectra (f) and (g); 12 550 (5 Hz) for spectrum (i); 100 800 (20 Hz) for spectra (j) and (k). Sample conditions were: (a) 1.4 ml of 2.4 mM Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin at pH 7.07; (b) 1.5 ml of 2.3 mM Z-Gly-Gly-Phe-CH₂-subtilisin at pH 7.07; (c) difference spectrum, (a) – (b); (d) 1.0 ml of 3.0 mM Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin at pH 7.01; (e) 1.0 ml of 2.3 mM alkali-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin at pH 6.71; (f) 1.1 ml of 2.8 mM alkali-denatured Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin at pH 7.01; (g) 1.2 ml of 2.5 mM alkali-denatured Z-Gly-Gly-Phe-CH₂-subtilisin at pH 7.05; (h) difference spectrum, (f) – (g); (i) 2.2 ml of 2.0 mM SDS-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin at pH 6.99; (j) 1.1 ml of 2.1 mM SDS-denatured Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin at pH 6.89; (k) 1.1 ml of 2.0 mM SDS-denatured Z-Gly-Gly-Phe-CH₂-subtilisin at pH 6.89; (l) difference spectrum, (j) – (k). All samples contained 20% (v/v) $^2\text{H}_2\text{O}$, 50 mM glycine, 50 mM glycyglycine and 2.5 mM potassium phosphate. The SDS-denatured samples contained 5% (w/w) SDS. The signals at 14.6 p.p.m., 23.5 p.p.m., 26.5 p.p.m., 30.8 p.p.m., 32.9 p.p.m. and 70.0 p.p.m. in spectra (i), (j) and (k) are due to SDS. The signals at 40.2 p.p.m., 41.5 p.p.m. and 44.1 p.p.m. in spectra (d), (e) and (i) are due to glycine and glycyglycine, which were added to all samples to ensure constant pH values. These signals were not clearly resolved in the spectra produced using a 40 Hz or 20 Hz exponential weighting factor.

creased according to a $\text{p}K_a$ of 5.58 (curve C in Figure 2), which is essentially the same as the $\text{p}K_a$ determined from the titration shift of the ^{13}C -enriched carbonyl carbon of the alkali-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin derivative (Table 1). Both the $\text{p}K_a$ and the negative titration shift are similar to those observed for the α -methylene carbon of alkali-denatured samples of δ -chymotrypsin- and trypsin-chloromethane derivatives (Table 1). The pH-dependent changes in the chemical shifts of the ^{13}C -NMR signals from the alkali-denatured/autolysed

Figure 2 pH titration of the signals due to the ^{13}C -enriched carbon atoms in denatured and intact Z-Gly-Gly-Phe-CH₂-subtilisin derivatives

Acquisition parameters and sample conditions were as described in the Materials and methods section. The continuous lines A, B and C were calculated using eqn. (1):

$$\delta = \frac{S_1}{1 + \frac{K_a}{[H]}} + \frac{S_2}{1 + \frac{[H]}{K_a}} \quad (1)$$

●, Curve A was calculated for the intact Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin derivative using the fitted parameters $\text{p}K_a = 7.04 \pm 0.07$, $S_1 = 56.10 \pm 0.04$ p.p.m. and $S_2 = 60.06 \pm 0.03$ p.p.m. ▲, Curve B was calculated for the alkali-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin derivative using the fitted parameters $\text{p}K_a = 5.66 \pm 0.09$, $S_1 = 202.49 \pm 0.05$ p.p.m. and $S_2 = 205.40 \pm 0.03$ p.p.m. ■, Curve C was calculated for the alkali-denatured Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin derivative using the fitted parameters $\text{p}K_a = 5.58 \pm 0.13$, $S_1 = 56.16 \pm 0.04$ p.p.m. and $S_2 = 54.54 \pm 0.02$ p.p.m.

samples of trypsin-, chymotrypsin- and subtilisin-(chloromethane inhibitor) derivatives were reversible. Also, no new signals at ~ 100 p.p.m. characteristic of the intact 2- ^{13}C -enriched inhibitor derivatives were detected when the pH of these alkali-denatured/autolysed inhibitor derivatives was decreased to acid pH values. Therefore these alkali-denatured/autolysed proteins could not re-adopt their native structures at acid pH values. The α -methylene carbons of alkali-denatured samples of Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin had linewidths of 23.3 ± 1.6 Hz, while those of the intact Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin derivative had linewidths of 41.7 ± 1.7 Hz. The larger linewidth in the intact sample is expected if the α -methylene carbon has a greater motional freedom in the denatured derivative [14].

Heating a sample of Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin in 5% (w/w) SDS for 3 min at pH 6.89 resulted in loss of the signal at ~ 100 p.p.m. due to the sp^3 hybridized hemiketal carbon of the intact chloromethane inhibitor derivative (structures A and B in Scheme 1), and in the appearance of a new signal with a chemical shift of 202.85 p.p.m. (Figure 1i) characteristic of an sp^2

Scheme 1 Structures and chemical shifts of the intact and denatured enzyme-(chloromethane inhibitor) derivatives listed in Table 1

Table 1 Titration constants obtained by ^{13}C -NMR studies of trypsin-, chymotrypsin- and subtilisin-(chloromethane inhibitor) derivatives

KCl (0.4 M) was included in all samples of chymotrypsin. It was not present in samples of trypsin or subtilisin. Alkali-denatured samples were prepared by alkaline denaturation/autolysis; SDS-denatured samples were prepared by heating in the presence of SDS, as described in the Materials and methods section. δ_1 and δ_2 are the pH-independent chemical shifts at low and high pH respectively.

Enzyme	Inhibitor	State of enzyme-inhibitor derivative	Chemical shift (p.p.m.)			pK_a	Reference
			δ_1	δ_2	$\delta_2 - \delta_1$		
Subtilisin	Z-Gly-Gly-[1- ^{13}C]Phe-CH ₂ Cl	Intact	56.10	60.06	3.96	7.04	Present work
Trypsin	Z-[1- ^{13}C]Lys-CH ₂ Cl	Intact	54.84	59.39	4.55	7.99	14
δ -Chymotrypsin	Tos-[1- ^{13}C]Phe-CH ₂ Cl	Intact	55.10	59.50	4.40	8.99	12
Subtilisin	Z-Gly-Gly-[2- ^{13}C]Phe-CH ₂ Cl	Intact	98.87	103.59	4.72	6.92	13
Trypsin	Z-[2- ^{13}C]Lys-CH ₂ Cl	Intact	98.95	103.08	4.13	7.88	9
δ -Chymotrypsin	Tos-[2- ^{13}C]Phe-CH ₂ Cl	Intact	99.10	103.66	4.56	8.85	12
δ -Chymotrypsin	Z-Gly-Gly-[2- ^{13}C]Phe-CH ₂ Cl	Intact	98.47	103.17	4.70	8.92	13
δ -Chymotrypsin	Z-[2- ^{13}C]Phe-CH ₂ Cl	Intact	98.79	103.58	4.79	9.42	13
Subtilisin	Z-Gly-Gly-[2- ^{13}C]Phe-CH ₂ Cl	Alkali-denatured	202.49	205.40	2.91	5.66	Present work
Trypsin	Z-[2- ^{13}C]Lys-CH ₂ Cl	Alkali-denatured	203.64	206.62	2.98	5.26	9
δ -Chymotrypsin	Z-Gly-Gly-[2- ^{13}C]Phe-CH ₂ Cl	Alkali-denatured	202.37	205.34	2.97	5.55	Present work
δ -Chymotrypsin	Z-[2- ^{13}C]Phe-CH ₂ Cl	Alkali-denatured	203.30	206.23	2.93	5.46	Present work
α -Chymotrypsin	Tos-[2- ^{13}C]Phe-CH ₂ Cl	Alkali-denatured	203.52	206.08	2.56	5.27	11
δ -Chymotrypsin	Z-Gly-Gly-[2- ^{13}C]Phe-CH ₂ Cl	SDS-denatured	200.49	203.61	3.12	6.51	Present work
Subtilisin	Z-Gly-Gly-[2- ^{13}C]Phe-CH ₂ Cl	SDS-denatured	200.52	203.98	3.46	6.71	Present work
Subtilisin	Z-Gly-Gly-[1- ^{13}C]Phe-CH ₂ Cl	SDS-denatured	56.17	54.64	-1.53	6.58	Present work
Subtilisin	Z-Gly-Gly-[1- ^{13}C]Phe-CH ₂ Cl	Alkali-denatured	56.16	54.54	-1.62	5.58	Present work
Trypsin	Z-[1- ^{13}C]Lys-CH ₂ Cl	Alkali-denatured	56.04	54.14	-1.90	5.36	14
δ -Chymotrypsin	Tos-[1- ^{13}C]Phe-CH ₂ Cl	Alkali-denatured	56.28	54.84	-1.44	5.36	12

hybridized carbonyl carbon (structures C and D in Scheme 1). This confirms that this treatment has produced denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin (structures C and D in Scheme 1). SDS-denatured samples of Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin (Figure 1j) and Z-Gly-Gly-Phe-CH₂-subtilisin (Figure 1k) were prepared at pH 6.89 and the difference spectrum (Figure 1l) contained a single signal at 55.17 p.p.m. due to the ^{13}C -enriched α -methylene carbon of the denatured Z-Gly-Gly-[1- ^{13}C]Phe-

CH₂-subtilisin derivative. The chemical shift of the α -methylene carbon in the SDS-denatured Z-Gly-Gly-[1- ^{13}C]Phe-CH₂-subtilisin derivative decreased as the pH increased according to a pK_a of 6.58, which is essentially the same as the pK_a values obtained from the SDS-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂-subtilisin derivative and the SDS-denatured Z-Gly-Gly-[2- ^{13}C]Phe-CH₂- δ -chymotrypsin derivative (Table 1). Therefore denaturation by SDS or alkali can be used to prepare denatured

Scheme 2 Ionizations with subtilisin–(chloromethane inhibitor) derivatives

Im, imidazolium cation; OE, O^γ of serine-221 of subtilisin BPN'.

samples of chloromethane inhibitor derivatives. However, the pK_a values obtained from the titration shifts of SDS-denatured Z-Gly-Gly-[2-¹³C]Phe-CH₂-subtilisin and Z-Gly-Gly-[2-¹³C]Phe-CH₂-δ-chymotrypsin are both approx. 1 pK_a unit higher than the pK_a values obtained with samples subjected to alkaline denaturation/autolysis (Table 1). The titration shifts of the SDS-denatured chloromethane inhibitor derivatives are also larger than those of other alkali-denatured samples (Table 1). These increases in titration shift and pK_a values in SDS-denatured samples may reflect differences in the environments of the alkylated histidine residue resulting from hydrolysis of peptide bonds during alkali denaturation/autolysis, and they may also be due to the presence of SDS in SDS-denatured samples.

Assignment of the titration shifts and pK_a values associated with the 1-¹³C- and 2-¹³C-enriched Z-Gly-Gly-Phe-CH₂-subtilisin derivatives

Using model compounds, it has been shown that the α-methylene carbons of N-alkylated imidazoles undergo a characteristic negative titration of approx. –2 p.p.m. on deprotonation of the imidazolium ion [12,14]. With both the denatured 1-¹³C-enriched trypsin- and chymotrypsin–(chloromethane inhibitor) derivatives the ¹³C-enriched α-methylene carbon has a negative titration shift (Table 1) and a pK_a of 5.36, which confirms that the inhibitor has alkylated the active-site histidine [12,14]. However, the positive titration shifts of ~4.5 p.p.m. of the α-methylene carbons of the intact trypsin- and chymotrypsin–inhibitor derivatives (Table 1) cannot be assigned to ionization of the active-site histidine residue. Studies of model compounds show that such large positive titration shifts occur at the α-methylene carbon when the oxyanion is formed [12]. This, plus the fact that the pK_a values associated with the α-methylene titration shifts are the same as those observed for oxyanion formation with the equivalent 2-¹³C-enriched derivatives (Table 1), confirms that the α-methylene titration shifts are due to oxyanion formation [12,14]. As no negative α-methylene titration shift was detected up to pH

~10.5, it was concluded that the pK_a of His-57 is >11 in the trypsin- and chymotrypsin–chloromethane derivatives [12,14].

The negative titration shift for the ¹³C-enriched α-methylene carbon of the alkali- and SDS-denatured Z-Gly-Gly-[1-¹³C]Phe-CH₂-subtilisin derivative (Table 1) confirms that the active-site histidine residue has been alkylated. The large positive titration shift of the α-methylene carbon of the intact Z-Gly-Gly-[1-¹³C]Phe-CH₂-subtilisin derivative (Table 1) shows that this titration shift is not due to ionization of the alkylated imidazolium ion of the active-site histidine, but instead it must reflect oxyanion formation [12]. This assignment is supported by the fact that the pK_a of this titration shift is essentially the same as that obtained for oxyanion formation with the Z-Gly-Gly-[2-¹³C]Phe-CH₂-subtilisin derivative (Table 1). As no negative titration shift was detected up to pH 11.5 with the Z-Gly-Gly-[1-¹³C]Phe-CH₂-subtilisin derivative (curve A in Figure 2), we conclude that in the Z-Gly-Gly-Phe-CH₂-subtilisin derivatives the pK_a of the imidazolium ion of the alkylated active-site histidine residue is >12. Therefore the oxyanion pK_a (pK_2 in Scheme 2) is ~7.0 and the histidine pK_a (pK_4 in Scheme 2) is >12.

Stabilization of zwitterionic tetrahedral adducts in enzyme–(chloromethane inhibitor) derivatives

Our results show that in the subtilisin–(chloromethane inhibitor) derivatives the pK_a of the active-site histidine is greater than that of the oxyanion. Therefore, like trypsin and chymotrypsin, subtilisin preferentially stabilizes the zwitterionic tetrahedral adduct (species iii in Scheme 2) in chloromethane inhibitor derivatives. K_T ($K_T = K_1/K_2 = K_3/K_4$ in Scheme 2) is the tautomeric ratio of the neutral species (species ii in Scheme 2) to the zwitterionic species (species iii in Scheme 2). However, as the zwitterionic tautomer predominates, $K_T \ll 1$. Therefore, as $K_T = K_1/K_2$, then $pK_1 \gg pK_2$ and so $pK_1 \gg 7$. We have calculated that for analogous low-molecular-mass model compounds in water the neutral tautomer predominates (species ii in Scheme 2), and the pK_1 (Scheme 2) of the imidazolium cation and the pK_2 of the oxyanion will be 6.7 and 10.7 respectively; hence $K_T = 10000$ [12]. Therefore, as $K_T \ll 1$ in the subtilisin–(chloromethane inhibitor) derivative, then this derivative must be at least 10000 times more effective at stabilizing the zwitterionic tautomer than the analogous model compound in water. Essentially the same result has been obtained [12,13] with chymotrypsin and trypsin, which are mammalian enzymes belonging to the chymotrypsin superfamily of homologous enzymes. This shows that, as a result of convergent evolution, both the bacterial serine proteinase subtilisin BPN' and the mammalian serine proteinases trypsin and δ-chymotrypsin preferentially stabilize zwitterionic tetrahedral adducts (species iii in Scheme 2).

Formation of tetrahedral intermediates during catalysis by the serine proteinases

Studies on model compounds suggest that in chloromethane inhibitor adducts an interaction between His-57 and Asp-102 is required to raise the pK_a of His-57 so that $pK_1 > pK_2$ and the zwitterionic tetrahedral adduct predominates [12,23]. It has been suggested that in catalytic tetrahedral intermediates, and in the tetrahedral adducts of chloromethane inhibitor derivatives, the substrate or inhibitor respectively could (1) lock the histidine in position, (2) desolvate the solvent-exposed N-3 of His-57, and (3) expel water from the active site [12,23a]. Any or all of these factors could contribute to raising the pK_a of the imidazolium cation in these adducts [12]. In the free enzyme the pK_a of the hydroxy group of Ser-195 is expected to be 15–16, while that of His-57 is ~7 [24,25]. Therefore, if substrate binding raises the

pK_a of His-57, this should enhance the nucleophilicity of the hydroxy group of Ser-195 by increasing the efficiency of the general-base-catalysed removal of the proton of the hydroxy group of serine by the imidazole group of His-57.

Recently it has been claimed [26,27] that the chemical shift values of 16.5–18.7 p.p.m. [28–30] for the N-1 hydrogen-bonded proton of His-57 show that this proton is involved in a low-barrier hydrogen bond when the imidazolium ion is formed both in the free enzyme and also in tetrahedral adducts. Low-barrier hydrogen bonds are short strong hydrogen bonds which are formed when the pK_a values of the two heteroatoms bridged by the hydrogen bonds are close to equality [31,32]. Therefore it has been suggested [26,27] that in the free enzyme the pK_a of Asp-102 must be raised to a value of ~ 7 . This would also imply that when tetrahedral adducts are formed the pK_a of Asp-102 would be raised to an even higher value so that it was similar to that of the imidazolium ion of His-57 in the tetrahedral adduct. In their mechanism Frey et al. [26] propose that the imidazole of His-57 abstracts a proton from the hydroxy group of Ser-195 to form a tetrahedral adduct. The protonated imidazolium ion which is produced then forms a low-barrier hydrogen bond with Asp-102. As it is proposed that the low-barrier hydrogen bond is formed after tetrahedral adduct formation, then this mechanism does not explain how the nucleophilicity of the hydroxy group of Ser-195 is enhanced. It has been suggested that formation of a low-barrier hydrogen bond between His-57 and Asp-102 stabilizes the imidazole-CO₂H portion of the catalytic triad [26] and the formation of a tetrahedral intermediate [27]. All of the three mechanisms outlined earlier [12] raised the pK_a of His-57 by enhancing the charge-charge interaction between the imidazolium cation of His-57 and the carboxylate anion of Asp-102, and so could be responsible for the formation of a low-barrier hydrogen bond. If a low-barrier hydrogen bond is formed when a tetrahedral adduct is formed then the pK_a of Asp-102 must be raised to a value similar to that of His-57. This result is expected if substrate binding decreases the effective dielectric constant between His-57 and Asp-102, as this should raise the pK_a of Asp-102 (a neutral acid) but have little effect on the pK_a of His-57 (a cationic acid) [33].

However, it has also been argued [34] that, while hydrogen bonds are important in enzyme catalysis, low-barrier hydrogen bonds destabilize ionic transition states relative to asymmetrical hydrogen bonds as well as the corresponding case in water and thus lead to 'anticatalysis'. These authors suggest that the protein decreases the pK_a of Asp-102 by providing a network of hydrogen bonds to it. IR studies of trypsin also support this suggestion, as they have shown that both in the free enzyme and in complexes with both soybean trypsin inhibitor and bovine pancreatic trypsin inhibitor a strong hydrogen bond exists between His-57 and Asp-102 at pH 7, with His-57 being protonated and Asp-102 being ionized [35]. The predominance of this tautomer is ascribed to strong specific interactions of Asp-102 and of the hydrogen bond with their environments [35]. Therefore it appears that the enzyme stabilizes the ionized form of Asp-102 which is required to raise the pK_a (pK_1 of Scheme 2) of His-57. This plus oxyanion stabilization ensures that $pK_1 > pK_2$ (Scheme 2), which enables the enzyme to preferentially stabilize the zwitterionic form of the tetrahedral adduct (species iii in Scheme 2). We suggest that substrate binding raises the pK_a of His-57 by

enhancing the effectiveness of its electrostatic/hydrogen-bond interaction with Asp-102 by any or all of the three mechanisms we outlined previously [12]. This increase in pK_a decreases the activation energy for formation of the tetrahedral intermediate by enabling the imidazole group to be a more efficient general base catalyst for increasing the nucleophilicity of the serine hydroxy group. Once formed it is the zwitterionic tetrahedral intermediate (species iii in Scheme 2), consisting of the oxyanion and the imidazolium cation, which is stabilized for optimal catalytic efficiency. The oxyanion is stabilized by its electrostatic interaction with His-57 and by its solvation by hydrogen bonding in the oxyanion hole [13], and the imidazolium cation is stabilized, in part at least, by its interaction with Asp-102 and the oxyanion.

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