¹³C-NMR study of the inhibition of δ -chymotrypsin by a tripeptide-glyoxal inhibitor

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A new inhibitor, Z-Ala-Pro-Phe-glyoxal (where Z is benzyl-oxycarbonyl), has been synthesized and shown to be a competitive inhibitor of δ -chymotrypsin, with a K_i of 25 ± 8 nM at pH 7.0 and 25 °C. Z-Ala-Pro-[1-¹³C]Phe-glyoxal and Z-Ala-Pro-[2-¹³C]Phe-glyoxal have been synthesized, and ¹³C-NMR has been used to determine how they interact with δ -chymotrypsin. Using Z-Ala-Pro-[2-¹³C]Phe-glyoxal we have detected a signal at 100.7 p.p.m. which we assign to the tetrahedral adduct formed between the hydroxy group of Ser-195 and the ¹³C-enriched keto-carbon of the inhibitor. This signal is in a pH-dependent slow exchange with a signal at 107.6 p.p.m. which depends on a pK_a of ~ 4.5 , which we assign to oxyanion formation. Thus we are the first to detect an oxyanion pK_a in a reversible chymotrypsin–inhibitor complex. A smaller titration shift of 100.7 p.p.m. to 103.9 p.p.m. with a pK_a of ~ 5.3 is also detected due to a rapid exchange process. This pK_a is also detected with the Z-Ala-Pro-[1-¹³C]Phe-glyoxal inhibitor and gives a larger titration shift of

91.4 p.p.m. to 97.3 p.p.m., which we assign to the ionization of the hydrated aldehyde hydroxy groups of the enzyme-bound inhibitor. Protonation of the oxyanion in the oxyanion hole decreases the binding efficiency of the inhibitor. From this decrease in binding efficiency we estimate that oxyanion binding in the oxyanion hole reduces the oxyanion pK_a by 1.3 pK_a units. We calculate that the pK_a s of the oxyanions of the hemiketal and hydrated aldehyde moieties of the glyoxal inhibitor are both lowered by 6.4–6.9 pK_a units on binding to chymotrypsin. Therefore we conclude that oxyanion binding in the oxyanion hole has only a minor role in decreasing the oxyanion pK_a . We also investigate how the inhibitor breaks down at alkaline pH, and how it breaks down at neutral pH in the presence of chymotrypsin.

Key words: Cannizzaro reaction, oxyanion, pK_a , protease.

INTRODUCTION

Glyoxals, or α -keto- β -aldehydes (RCOCHO), are reactive compounds which have been shown to be cytotoxic [1,2]. They have been used as radiosensitizers of cells [3] and as arginine reactivity probes [4].

Several specific substrate-derived glyoxal inhibitors of proteases have been synthesized [5] and shown to be potent inhibitors of chymotrypsin [6,7], cathepsin B [7,8], cathepsin L [8], cathepsin S [9] and the proteasome [10]. However, there have been no studies on the structure and stability of the enzyme–glyoxal-inhibitor complexes, or of the mechanism of inhibition. Therefore one of the main aims of the present work is to undertake such studies with chymotrypsin.

Catalysis by the serine proteases is thought to proceed via a tetrahedral intermediate whose oxyanion is stabilized by hydrogen bonding in the oxyanion hole [11,12]. There is considerable interest in determining how the oxyanion hole contributes to oxyanion stabilization [13–18]. We have undertaken an extensive ¹³C-NMR study of oxyanion stabilization in trypsin [19–23], subtilisin [15,16,23,24] and chymotrypsin [15,22,23,25] using substrate-derived chloromethane inhibitors. However, one of the limitations of these inhibitors is that they are irreversible inhibitors which alkylate the active-site histidine residue. Therefore they cannot be used to determine how binding energy is utilized to lower the oxyanion pK_a . Also, there is always the possibility that, as a result of the alkylation of the active-site histidine residue, the oxyanion may not be in its optimal position in enzyme–chloromethane-inhibitor derivatives [26,27]. Substrate-derived glyoxal inhibitors are reversible inhibitors, and we would expect the α -keto carbon of the glyoxal to occupy the

same position as the carbonyl of a substrate. Therefore we predict that glyoxal inhibitors will form tetrahedral adducts which closely resemble the tetrahedral intermediate formed in catalysis. An earlier study utilized a fluoroketone inhibitor which formed a tetrahedral adduct with chymotrypsin [26]. However, this was not a good system for studying oxyanion stabilization, because an oxyanion pK_a value could not be determined and because introducing an unnatural extremely electronegative fluorine could give the oxyanion abnormal properties, such as a very low pK_a . Therefore in the present work we hope that substrate-derived glyoxal inhibitors will enable us to determine oxyanion pK_a values and provide a useful system for studying how binding energy is used to stabilize the oxyanion.

MATERIALS AND METHODS

Materials

N- α -Z-L-alanine (where Z is benzyloxycarbonyl) and L-proline methyl ester were obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, U.K.). L-[1-¹³C]Phenylalanine (99 atom %) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.) and from CDN Isotopes (Pointe-Claire, Quebec, Canada). All other chemicals used were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Synthesis of Z-Ala-Pro-Phe and Z-Ala-Pro-[1-¹³C]Phe

These compounds were synthesized in a stepwise manner using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride as a coupling reagent, as described by Sheehan and Ledis [28].

Abbreviations used: T_1 , spin lattice relaxation time; Z, benzyloxycarbonyl.

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The yield of Z-Ala-Pro-Phe was 0.280 g (0.600 mmol; 90 %). ^{13}C -NMR analysis gave the following data. *Trans* form (83.2 %): δ ($[\text{H}]\text{chloroform}$) 17.69 (1C, CHCH_3), 24.79 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 27.33 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 37.22 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 47.31 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 48.15 (1C, CHCH_3), 53.26 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 60.24 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 66.81 (1C, $\text{C}_6\text{H}_5\text{CH}_2$), 126.94–129.36 (10C, $\text{CH}=\text{CH}$), 136.10–136.33 (2C, $\text{CH}=\text{C}=\text{}$), 155.98 (1C, O-CO-NH), 170.51 and 173.67 (2C, CO-NH), 173.55 (1C, COOH). *Cis* form (16.8 %): δ ($[\text{H}]\text{chloroform}$) 16.74 (1C, CHCH_3), 21.49 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 31.47 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 37.74 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 46.98 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 49.19 (1C, CHCH_3), 54.28 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 60.76 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 67.24 (1C, $\text{C}_6\text{H}_5\text{CH}_2$), 126.94–129.36 (10C, $\text{CH}=\text{CH}$), 136.10–136.33 (2C, $\text{CH}=\text{C}=\text{}$), 155.98 (1C, O-CO-NH), 171.32 and 172.65 (2C, CO-NH), 173.42 (1C, COOH). Chemical analysis: calculated for $\text{C}_{25}\text{H}_{29}\text{N}_3\text{O}_6$: C, 62.9 %; H, 6.2 %; N, 8.6 %; O, 22.3 %; found: C, 64.2 %; H, 6.2 %; N, 9.0 %; O, 20.6 %.

The ^{13}C -NMR spectrum of Z-Ala-Pro-[1- ^{13}C]Phe in $[\text{H}]\text{chloroform}$ contained a single signal at 173.57 p.p.m. due to the ^{13}C -enriched carbon atom.

Conversion of Z-Ala-Pro-Phe into Z-Ala-Pro-Phe-glyoxal, Z-Ala-Pro-Phe-[1- ^{13}C]glyoxal and Z-Ala-Pro-Phe-[2- ^{13}C]glyoxal

Z-Ala-Pro-Phe was converted into Z-Ala-Pro-Phe-diazoketone using diazomethane and the mixed anhydride method described by Coggins et al. [29]. The diazoketone was converted into a glyoxal using a procedure modified from that described by Ihmels et al. [30]. A 200 mg (0.407 mmol) quantity of Z-Ala-Pro-Phe-diazoketone was oxidized by dissolving it in a 5 ml solution of 0.1 M dimethyldioxirane in acetone and stirring until no more nitrogen was evolved. The reaction mixture was then moistened by the addition of $\sim 0.25\%$ (v/v) water and the acetone was removed by evaporation under reduced pressure. The moist compound was then dissolved in $[\text{H}_6]\text{DMSO}$.

^{13}C -NMR analysis gave the following data. *Trans* form (90.9 %): δ ($[\text{H}_6]\text{DMSO}$) 16.95 (1C, CHCH_3), 24.54 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 29.03 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 35.41 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 46.79 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 48.16 (1C, CHCH_3), 55.55 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 59.43 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 65.59 (1C, $\text{C}_6\text{H}_5\text{CH}_2$), 89.05 [1C, CO-CH(OH) $_2$], 126.52–129.69 (10C, $\text{CH}=\text{CH}$), 137.19–137.97 (2C, $\text{CH}=\text{C}=\text{}$), 155.92 (1C, O-CO-NH), 171.33–171.83 (2C, CO-NH), 205.82 [1C, COCH(OH) $_2$]. *Cis* form (9.1 %): δ ($[\text{H}_6]\text{DMSO}$) 16.55 (1C, CHCH_3), 21.24 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 31.34 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 34.91 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 46.55 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 48.45 (1C, CHCH_3), 55.94 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 60.28 (1C, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 65.91 (1C, $\text{C}_6\text{H}_5\text{CH}_2$), 89.33 [1C, CO-CH(OH) $_2$], 126.52–129.69 (10C, $\text{CH}=\text{CH}$), 137.19–137.97 (2C, $\text{CH}=\text{C}=\text{}$), 154.95 (1C, O-CO-NH), 171.17–171.54 (2C, CO-NH), 205.38 [1C, COCH(OH) $_2$].

Z-Ala-Pro-Phe-[1- ^{13}C]glyoxal and Z-Ala-Pro-Phe-[2- ^{13}C]glyoxal were synthesized by the same procedure, except that *N*-[^{13}C]methyl-*N*-nitrosotoluene-*p*-sulphonamide was used to generate the diazomethane used in the synthesis of Z-Ala-Pro-Phe-[1- ^{13}C]glyoxal, and [1- ^{13}C]Phe ethyl ester was used for the synthesis of Z-Ala-Pro-Phe-[2- ^{13}C]glyoxal. The signals from the ^{13}C -enriched carbons in water and in DMSO solvents are described and assigned in the Results and discussion section.

Estimation of the amounts of *cis* and *trans* isomers in Z-Ala-Pro and Z-Ala-Pro-Phe

T_1 (spin lattice relaxation time) values of the proline carbons of Z-Ala-Pro-Phe were estimated by the inversion recovery method. The signals due to the *cis* (31.47 p.p.m.) and *trans* (27.33 p.p.m.)

forms of the β -carbon of proline in Z-Ala-Pro-Phe in $[\text{H}]\text{chloroform}$ had T_1 values of 0.198 ± 0.021 s and 0.244 ± 0.008 s respectively, while the signals due to the *cis* (21.49 p.p.m.) and *trans* (24.79 p.p.m.) forms of the γ -carbon of proline in Z-Ala-Pro-Phe had T_1 values of 0.243 ± 0.008 s and 0.279 ± 0.008 s respectively. Therefore, in order to quantify the amounts of the *cis* and *trans* forms, an interpulse delay of 5 s was used with no proton decoupling to ensure there were no nuclear Overhauser effects or relaxation effects on the signals. The same approach was used to estimate the amounts of *cis* and *trans* forms in Z-Ala-Pro. Similar amounts of *cis* and *trans* isomers ($\pm 1\%$) were obtained if decoupled spectra were used, showing that the nuclear Overhauser effect was complete, as expected for molecules with $M_r > 300$ [31]. Therefore, to improve the signal/noise ratio with less concentrated samples of Z-Ala-Pro-Phe-glyoxal in $[\text{H}]\text{DMSO}$, decoupled spectra were used to estimate the amounts of the *cis* and *trans* isomers.

Enzyme solutions

δ -Chymotrypsin (salt free, from bovine pancreas) was obtained from Sigma Chemical Co., and the amount of fully active protein (83 %) was determined as described by Finucane et al. [25].

Inhibition of δ -chymotrypsin by Z-Ala-Pro-Phe-glyoxal

The inhibition of the δ -chymotrypsin-catalysed hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by Z-Ala-Pro-Phe-glyoxal was studied at 25 °C in 0.1 M buffer (potassium formate, pH 3.31 and 4.02; potassium acetate, pH 5.01; potassium phosphate, pH 6.05 and 7.01) containing 3.3 % (v/v) DMSO. K_i values for the inhibitor were determined using the method of Henderson [32]. Stock solutions of 24 mM substrate and 0.192 mM inhibitor were dissolved in DMSO. Final concentrations of enzyme, substrate and inhibitor were in the ranges 2–125 nM, 0.03–1.00 mM and 0.03–4.00 μM respectively.

NMR spectroscopy

NMR spectra at 11.75 T were recorded with a Bruker Avance DRX 500 standard-bore spectrometer operating at 125.7716 MHz for ^{13}C nuclei. Unless stated otherwise 10 mm-diameter sample tubes were used. The spectral conditions for the samples of chymotrypsin inhibited by Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal at 11.75 T were: 32 768 time-domain data points; spectral width 240 p.p.m.; acquisition time 0.541 s; 7.7 s relaxation delay time; 90° pulse angle; 256 or 512 transients recorded per spectrum. Waltz-16 composite pulse ^1H decoupling with a BLARH100 amplifier was used with 16 dB attenuation during the acquisition time and 34 dB attenuation during the relaxation delay to minimize dielectric heating but maintain the nuclear Overhauser effect. All spectra were transformed using an exponential weighting factor of 30, 50 or 100 Hz. For samples of chymotrypsin inhibited by Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal at 11.75 T, the spectral conditions were: 8192 time-domain data points; spectral width 240 p.p.m.; acquisition time 0.135 s; 0.6 s relaxation delay time; 90° pulse angle; 512–4096 transients recorded per spectrum. All spectra were zero-filled to give 32 768 data points before being transformed.

Both ^1H and ^{13}C chemical shifts are quoted relative to tetramethylsilane at 0.00 p.p.m. In aqueous solutions the chemical shift of the α -carbon of glycine was used as a chemical shift reference, as described previously [25]. For non-aqueous solvents either 10 % tetramethylsilane was used as an internal standard or an appropriate solvent signal was used as a secondary reference [31].

All aqueous samples contained 10% (v/v) $^2\text{H}_2\text{O}$ to obtain a deuterium lock signal, as well as 10 mM potassium phosphate buffer to help maintain stable pH values during pH titrations.

Calculation of pK_a values

The pK_a values of hemiketal hydroxy groups were calculated using the relationship $pK_a = -7.39\Sigma\sigma_1 + 15.472$ [33]. The pK_a values of hydrate hydroxy groups were calculated using the relationship $pK_a = -1.42\Sigma\sigma^* + 14.4$ [34]. The σ_1 values of the α -carbon atoms of the S_1 amino acids are all very similar [15], and a value of $\sigma_1 = 0.13$ or $\sigma^* = 0.81$ [19] was used for all inhibitors. A value of $\sigma^* = 1.34$ was used for the hydroxy substituent [34]. All other substituent constants (σ^* and σ_1) used were those quoted previously [19].

RESULTS AND DISCUSSION

Inhibition of δ -chymotrypsin by Z-Ala-Pro-Phe-glyoxal

The inhibition of the chymotrypsin-catalysed hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by Z-Ala-Pro-Phe-glyoxal at pH 7.0 was analysed using eqn (1) [32], where $[E_t]$, $[S_t]$ and $[I_t]$ are the total concentrations of enzyme, substrate and inhibitor respectively, and v_o and v_i are initial rates of substrate catalysis in the absence and presence respectively of the inhibitor:

$$[I_t]/(1 - v_i/v_o) = (K_i\{([S_t] + K_m)/K_m\}v_o/v_i) + [E_t] \quad (1)$$

Plots of $[I_t]/(1 - v_i/v_o)$ against v_o/v_i were linear (Figure 1a), and a plot of the slopes of these graphs against the substrate concentration was linear (Figure 1a, inset), demonstrating that the inhibition was competitive [32], with $K_i = 25 \pm 8$ nM at pH 7.0 and 25 °C. This inhibitor is ~ 30 -fold more effective than Z-Pro-Phe-glyoxal at pH 7.4 and 37 °C, which is the most effective glyoxal inhibitor of chymotrypsin reported previously [7]. Z-Phe-glyoxal [6], Z-Pro-Phe-glyoxal [7] and Z-Ala-Pro-Phe-glyoxal have K_i values of 65, 0.85 and 0.025 μM respectively, which shows that binding in the S_2 and S_3 subsites of chymotrypsin makes a significant contribution to the binding of this glyoxal inhibitor to the enzyme. On decreasing the pH, the K_i value increased from 25.8 ± 18.4 nM to 565.0 ± 33.6 nM, according to a pK_a of 4.4 ± 0.1 (Figure 1b). Chymotrypsin activity (k_{cat} or k_{cat}/K_m) increases with a pK_a of 6–7 due to ionization of the active-site histidine, but the catalytic activity (k_{cat}/K_m) of the free enzyme decreases with a pK_a of ~ 8.8 due to a conformational change resulting from the ionization of the Ile-16–Asp-194 ion pair, which leads to catalytic inactivation [25]. However, binding in the S_1 subsite prevents this conformational change by raising the pK_a of the Ile-16–Asp-194 ion pair to > 11 [25]. In the present work δ -chymotrypsin is saturated with the Z-Ala-Pro-Phe-glyoxal inhibitor, which binds in the S_1 subsite, locking it in the active conformation between pH 3.3 and 7.0. Binding of the inhibitor is also expected to raise the histidine pK_a , promoting oxyanion formation if a tetrahedral adduct is formed [22].

^{13}C -NMR signals obtained from Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal and Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal in water

In fully aqueous media at pH 3.3, the α -keto carbon of Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal was $\sim 50\%$ hydrated, with signals at 206.8 and 96.5 p.p.m. (see Figure 3a) due to the α -keto carbon

Scheme 1 Structures and chemical shifts of Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal and Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal in water

See the text for details.

Figure 1 Inhibition of δ -chymotrypsin by Z-Ala-Pro-Phe-glyoxal

(a) Inhibition of the δ -chymotrypsin-catalysed hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SucAAPpNA) by Z-Ala-Pro-Phe-glyoxal at pH 7.01 and 25 °C: effect of changing the concentration of substrate at a fixed concentration of enzyme. The continuous lines are the fitted values for substrate concentrations of 40 ($\circ$), 100 ($\square$), 200 ($\triangle$), 300 ($\bullet$) and 400 ($\blacksquare$) μM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide using eqn (1), as described in the Results section. An enzyme concentration of 2 nM was used. All other experimental details are described in the Materials and methods section. Inset: the slopes $\{K_i([S_t] + K_m)/K_m\}$ of the plots of $[I_t]/(1 - v_i/v_o)$ against v_o/v_i are plotted against substrate concentration, and the intercept on the ordinate gives a K_i of 25 ± 8 nM. (b) Effect of pH on the binding of Z-Ala-Pro-Phe-glyoxal to δ -chymotrypsin. The continuous line was calculated using eqn (2) and the fitted parameters $K_2 = 25.8 \pm 18.4$ nM, $K_1 = 565.0 \pm 33.6$ nM and $pK_a = 4.4 \pm 0.1$:

$$K_{i(\text{obs})} = K_1/(1 + K_a/[\text{H}]) + K_2/(1 + [\text{H}]/K_3) \quad (2)$$

Table 1 Titration constants obtained by ^{13}C -NMR for α -hydroxycarboxylic acid model compounds and the breakdown products of Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal and Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal

Compound	Structure	Carbon observed	δ (p.p.m.)			pK_a
			δ_1	δ_2	$\delta_2 - \delta_1$	
Lactic acid	$\text{CH}_3\text{CHOHCOOH}$	—COOH	179.49	183.28	3.79	3.68
Lactic acid	$\text{CH}_3\text{CHOHCOOH}$	—CHOH—	67.34	69.39	2.05	3.68
Lactic acid	$\text{CH}_3\text{CHOHCOOH}$	CH_3 —	20.17	20.94	0.77	3.67
2-Hydroxybutyric acid	$\text{CH}_3\text{CH}_2\text{CHOHCOOH}$	—COOH	178.94	182.34	3.40	3.71
2-Hydroxybutyric acid	$\text{CH}_3\text{CH}_2\text{CHOHCOOH}$	—CHOH—	72.25	74.33	2.08	3.71
2-Hydroxybutyric acid	$\text{CH}_3\text{CH}_2\text{CHOHCOOH}$	— CH_2 —	27.52	27.90	0.38	3.71
2-Hydroxybutyric acid	$\text{CH}_3\text{CH}_2\text{CHOHCOOH}$	CH_3 —	9.27	9.46	0.19	3.71
Breakdown product of:						
Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal			72.85	74.93	2.08	3.38
Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal			176.12	178.83	2.71	3.39
Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal			72.77	74.89	2.12	3.44 (major signal)
Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal			71.26	72.89	1.63	3.61 (minor signal)
Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal			176.08	178.82	2.74	3.46 (major signal)
Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal			175.50	178.12	2.62	3.41 (minor signal)
Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal + enzyme			72.95	75.04	2.09	3.37
Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal + enzyme			176.08	178.82	2.74	3.44
Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal + enzyme			176.04	178.30	2.26	3.23

Figure 2 ^{13}C -NMR spectra of Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal and Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal before and after their breakdown in the presence of alkali or δ -chymotrypsin

Acquisition and processing parameters were as described in the Materials and methods section, except that for spectra (a)–(f) an exponential weighting factor of 30 Hz was used. For spectra (a)–(c) the interpulse delay was 8 s and 256 transients were recorded; for spectra (d)–(f) the interpulse delay was 2 s and 512 transients were recorded. Sample conditions were: (a) 3.5 ml of 2 mM Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal at pH 5.04; (b) 3.7 ml of 1.9 mM Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal at pH 5.01 after incubation at pH ≥ 11 ; (c) 3.2 ml of 1.4 mM Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal at pH 5.01 after irreversible breakdown in the presence of 1 mM δ -chymotrypsin; (d) 3.5 ml of 1 mM Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal at pH 5.03; (e) 3.7 ml of 0.9 mM Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal at pH 5.02 after incubation at pH ≥ 11 ; (f) 3.9 ml of 1.3 mM Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal at pH 5.01 after irreversible breakdown in the presence of 1 mM δ -chymotrypsin.

(structure 1, Scheme 1) and its hydrate (structure 2, Scheme 1) respectively. The aldehyde carbon of Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal was fully hydrated in fully aqueous media at pH 3, with signals at 88.7 and 90.2 p.p.m. (see Figure 5a), which were assigned to structures 1 and 2 in Scheme 1 respectively. The intensity and chemical shifts of the signals from Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal and Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal were unchanged between pH 3 and pH 10. However, after incubation at pH ≥ 11 , the signals at 96.5 and 206.8 p.p.m. from Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal (Figure 2a) and the signals at 90.2 and 88.7 p.p.m. from Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal (Figure 2d) at pH 5.0 were replaced by new signals, which at pH 5.0 (Figures 2b and 2e) had chemical shifts of 178.7 p.p.m. (no directly bonded protons) and 74.8 p.p.m. (one directly bonded proton; J_{CH} 147.5 $\pm$ 0.2 Hz at pH 5.0). The proton coupling patterns, chemical shift values, titration shifts and pK_a values of these signals (Table 1) suggest that the glyoxals (structure 2a in Scheme 2) have undergone an internal Cannizzaro reaction under alkaline conditions [35], producing an α -hydroxycarboxylic acid (structure 3a in Scheme 2). The internal Cannizzaro reaction involves a hydride shift [35] from C-1 to C-2 (from structure 2a to structure 3a in Scheme 2), and so the 1- ^{13}C - and 2- ^{13}C -enriched glyoxals are each expected to give one signal, at ~ 178 p.p.m. and ~ 74 p.p.m. respectively, when they form α -hydroxycarboxylic acids. The fact that both signals are observed with both enrichments can be explained if a C-2 to C-1 alkyl shift (reaction from structure 2b to structure 3b in Scheme 2) also occurs.

Effect of pH on the ^{13}C -NMR spectra of δ -chymotrypsin inhibited by Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal and Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal

When Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal (Figure 3a) was added to δ -chymotrypsin at pH 3.3 (Figure 3b), a new signal at 100.7 p.p.m. ($T_1 = 6.3 \pm 0.5$ s at pH 3.3) was observed (Figure 3c) which had no directly bonded protons. Hemiketals have chemical shifts that are ~ 3 p.p.m. greater than those of their hydrates [22], and so we assign the signal at 100.7 p.p.m. (Figure 3c) to the hemiketal carbon of a tetrahedral adduct formed between the active-site hydroxy group of Ser-195 and the ^{13}C -enriched carbon of Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal (structure 2, Scheme 3). As the pH

Scheme 2 Mechanisms of the irreversible breakdown of the free and enzyme-bound glyoxal inhibitor

See the text for details. 'E' denotes enzyme.

increased, there was a progressive reversible increase in the chemical shift of this signal (Figure 3 and curve A in Figure 4) from 100.70 ± 0.05 to 103.94 ± 0.07 p.p.m., resulting from the ionization of a group with a pK_a of 5.23 ± 0.05 .

When Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal (Figure 5a) was incubated with δ -chymotrypsin (Figure 5b) at pH 3.3, a new signal at 91.4 p.p.m. ($T_1 = 0.56 \pm 0.04$ s at pH 3.3) was observed (Figure 5c) which had one directly bonded proton (J_{CH} 161.4 $\pm$ 0.2 Hz); this was assigned to the hydrated aldehyde carbon of the tetrahedral adduct (structure 2, Scheme 3). On increasing the pH, the chemical shift of the signal at 91.4 p.p.m. increased to 97.1 p.p.m. (Figure 5) according to a pK_a of 5.29 ± 0.03 (curve B, Figure 4). This rapid exchange process was attributed to the ionization of the hydroxy groups of the hydrated aldehyde group (structures 2 and 3 in Scheme 3), giving a titration shift of 5.99 p.p.m. (Table 2). We assume that the smaller titration shift (3.24 p.p.m.) at the hemiketal carbon, which depends on a pK_a of 5.23 ± 0.05 , also reflects the ionization of the hydroxy groups of the hydrated aldehyde group (structures 2 and 3 in Scheme 3). The intensity of the signal at 91.4–97.1 p.p.m. (Figure 5) due to the hydrated aldehyde of the inhibitor (structure 2 in Scheme 3) decreased as the pH was increased, and a new signal at ~ 205.5 p.p.m. due to the inhibitor aldehyde carbon (structure 4 in Scheme 3) was observed (Figures 5d–5j). The intensity of the signal at 100.70–103.94 p.p.m. also decreased with increasing pH, and there was a concomitant increase in the intensity of a new signal at 107.57 ± 0.19 p.p.m. (Figure 3).

Figure 3 Effect of pH on the ^{13}C -NMR signals from Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal in the presence of δ -chymotrypsin

Acquisition and processing parameters were as described in the Materials and methods section, except that for spectra (a)–(k) an exponential weighting factor of 50 Hz was used. Sample conditions were: (a) 3.5 ml of 2 mM Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal at pH 3.32; (b) 3.3 ml of 2.5 mM δ -chymotrypsin; (c) 3.5 ml of 2.8 mM Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal containing 2.4 mM δ -chymotrypsin at pH 3.32; (d)–(j) as (c), except that the pH was increased progressively as shown; (k) pH was decreased to 3.30 to give a 4.1 ml solution of 2.5 mM Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal containing 2.1 mM δ -chymotrypsin.

Scheme 3 Structures and chemical shifts of enzyme–glyoxal-inhibitor adducts and their breakdown products

See the text for details. 'E' denotes enzyme.

Figure 4 pH titration of the signals in the complexes formed between δ -chymotrypsin and Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal or Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal

Acquisition parameters and sample conditions were as described in the Materials and methods section. The continuous lines were calculated using eqn (3):

$$\delta_{\text{obs}} = S_1/(1 + K_a/[H]) + S_2/(1 + [H]/K_a) \quad (3)$$

(A) pH titration of the signal at 100.7–103.94 p.p.m. in the complex formed between δ -chymotrypsin and Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal. The continuous line was calculated using the fitted parameters $pK_a = 5.23 \pm 0.05$, $S_1 = 100.70 \pm 0.05$ p.p.m. and $S_2 = 103.94 \pm 0.07$ p.p.m. (B) pH titration of the signal at 91.37–97.36 p.p.m. in the complex formed between δ -chymotrypsin and Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal. The continuous line was calculated using the fitted parameters $pK_a = 5.29 \pm 0.03$, $S_1 = 91.37 \pm 0.05$ p.p.m. and $S_2 = 97.36 \pm 0.07$ p.p.m. (C) pH titration of the signal at 72.95–75.04 p.p.m. formed by the irreversible breakdown of the complex between δ -chymotrypsin and Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal. The continuous line was calculated using the fitted parameters $pK_a = 3.37 \pm 0.02$, $S_1 = 72.95 \pm 0.03$ p.p.m. and $S_2 = 75.04 \pm 0.01$ p.p.m.

Ionization of an acid can occur by either mechanism 1 or mechanism 2, or by a combination of the two mechanisms [19,36,37]:

Assuming a line width of 20 Hz in the absence of exchange, we estimate that, at pH 4.28 (Figure 3e), the slow exchange line broadening is ~ 19 Hz and ~ 33 Hz for the signals at ~ 100.7 p.p.m. and ~ 107.6 p.p.m. respectively. From these line broadenings we estimate that, if mechanism 1 predominates, then $k_{\text{H(off)}} = \sim 60 \text{ s}^{-1}$, $k_{\text{H(on)}} = \sim 2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($k_{\text{obs}} = \sim 104 \text{ s}^{-1}$ at pH 4.28) and $pK_a = 4.5$, whereas if mechanism 2 predominates then $k_{\text{OH(off)}} = \sim 104 \text{ s}^{-1}$, $k_{\text{OH(on)}} = \sim 3.3 \times 10^{11}$

Figure 5 Effect of pH on the ^{13}C -NMR signals from Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal in the presence of δ -chymotrypsin

Acquisition and processing parameters were as described in the Materials and methods section, except that for spectra (a)–(k) an exponential weighting factor of 100 Hz was used and 4096 transients were recorded per spectrum. Sample conditions were: (a) 3.5 ml of 1.0 mM Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal; (b) 3.0 ml of 2.0 mM δ -chymotrypsin; (c) 3.1 ml of 2.1 mM Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal containing 2.0 mM δ -chymotrypsin; (d)–(j) as (c), except that the pH was increased progressively as shown; (k) pH was decreased to 3.32 to give a 3.4 ml solution of 1.9 mM Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal containing 1.8 mM δ -chymotrypsin.

$\text{M}^{-1} \cdot \text{s}^{-1}$ ($k_{\text{obs}} = \sim 60 \text{ s}^{-1}$ at pH 4.28) and $pK_a = 4.5$. The diffusion-controlled rate constant is expected to be $\sim 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$, and so it is unlikely that mechanism 2 predominates. If, as expected, mechanism 1 predominates at pH values < 7 , then $k_{\text{H(on)}} = \sim 2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. Formation of the hemiketal oxyanion (structure 4 in Scheme 3) is expected to increase the electro-negativity at the inhibitor aldehyde carbon, promoting its dehydration at higher pHs [38]. Therefore we assign this slow ionization ($pK_a \sim 4.5$) to the formation of the oxyanion (structure 4 in Scheme 3), which we propose results in the concomitant dehydration of the aldehyde group. All of these pH-dependent changes were reversible.

However, the intensity of the signals from δ -chymotrypsin inhibited by Z-Ala-Pro-[2- ^{13}C]Phe-glyoxal (Figure 3) or Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal (Figure 5) decreased irreversibly with time ($t_{1/2} = 17.3 \pm 4.9$ h at pH 7) and increasing pH, and they were replaced by new signals with chemical shifts of 178.8 p.p.m. (Figure 2c) and 74.9 p.p.m. (Figure 2f) respectively at pH 5.0. The pK_a values, chemical shifts and titration shift of these signals are essentially the same as those of the signals observed when the free inhibitor breaks down irreversibly at alkaline pH (Table 1), which suggests that the same compound has been formed. At alkaline pH both the 1- ^{13}C - and 2- ^{13}C -enriched free inhibitors produced two new signals, at 178.7 p.p.m. and 74.8 p.p.m. (Figures 2b and 2e). However, in the presence of δ -chymotrypsin the 1- ^{13}C - and 2- ^{13}C -enriched inhibitors only produced one signal each, at 74.8 p.p.m. (Figure 2f) and 178.7 p.p.m. (Figure 2c) respectively. Therefore at alkaline pH, in the absence of

Table 2 Titration constants obtained by ^{13}C -NMR studies of subtilisin, trypsin and δ -chymotrypsin–chloromethane-inhibitor derivatives and chymotrypsin–glyoxal-inhibitor complexes

δ_1 and δ_2 are the pH-independent chemical shifts at low and high pH respectively.

Enzyme	Inhibitor	Chemical shift (p.p.m.)			pK_a
		δ_1	δ_2	$\delta_2 - \delta_1$	
Chymotrypsin	Z-Ala-Pro-[2- ^{13}C]Phe-CHO	100.70	103.94	3.24	5.23
Chymotrypsin	Z-Ala-Pro-[1- ^{13}C]Phe-CHO	91.37	97.36	5.99	5.29
Chymotrypsin	Z-Ala-Pro-[2- ^{13}C]Phe-CHO	100.70	107.57	6.87	~ 4.5
Chymotrypsin	Z-Gly-Ala-[2- ^{13}C]Phe-CH $_2$ Cl*	98.58	103.17	4.59	8.97
Chymotrypsin	Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl*	98.47	103.17	4.70	8.92
Subtilisin	Z-Gly-Ala-[2- ^{13}C]Phe-CH $_2$ Cl*	99.41	103.85	4.44	6.94
Subtilisin	Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl*	98.87	103.59	4.72	6.92
Trypsin	Z-[2- ^{13}C]Lys-CH $_2$ Cl*	98.95	103.08	4.13	7.88

* Data from [15,19,23].

Table 3 Oxyanion stabilization by subtilisin, trypsin and δ -chymotrypsin

pK_{ae} is the experimentally determined pK_a value of the oxyanion in the enzyme–inhibitor adduct, and pK_{aw} is the pK_a calculated if the oxyanion were in water.

Enzyme–inhibitor complex or derivative	pK_{aw} (water)	pK_{ae} (enzyme)	$pK_{ae} - pK_{aw}$	ΔG (kJ·mol $^{-1}$)
Structures 2 and 4 in Scheme 3*	10.9	4.5	−6.4	−36.5
Structures 2 and 3 in Scheme 3*	12.2	5.3	−6.9	−39.4
Acetyl-Leu-[2- ^{13}C]Phe-CF $_3$ –chymotrypsin†	9.1	≤ 4.9	≥ -4.2	≥ -24.0
Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ –chymotrypsin‡	11.9	8.9	−3.0	−17.1
Z-[2- ^{13}C]Lys-CH $_2$ –trypsin‡	11.9	7.9	−4.0	−22.8
Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ –subtilisin‡	11.9	6.9	−5.0	−28.5

* Present work; chymotrypsin–Z-Ala-Pro-Phe-glyoxal complex.

† Data from [26]; pK_{aw} is the pK_a of an appropriate model compound [26].

‡ Chloromethane inhibitor derivatives.

enzyme, both alkyl and hydride shifts occur at similar rates, while in the presence of δ -chymotrypsin the alkyl shift from C-2 to C-1 predominates over the hydride shift at pH < 7 (structure 1 to structure 2 in Scheme 2b). This can be explained if the aldehyde carbonyl oxygen (structure 1 in Scheme 2b) is in the same location as the peptide nitrogen atom of the leaving group in substrate catalysis, and so its protonation will be promoted by general acid catalysis by His-157, while the hydride shift will be prevented by tetrahedral adduct formation. The alkyl shift produces an acyl enzyme (structure 2 in Scheme 2b) which is hydrolysed by the normal catalytic mechanism to produce the same α -hydroxy acid (structure 3 in Scheme 2b) as is produced by the breakdown of the free inhibitor (Scheme 2a). Additional minor signals at ~ 178 and ~ 73 p.p.m. (Table 1) were observed when there was irreversible breakdown of the glyoxal in the presence or absence of enzyme. These signals had similar titration characteristics to the major signals, and could reflect the formation of diastereoisomers [39] or proline *cis*–*trans* isomers [40,41] of the α -hydroxy acid breakdown product.

Oxyanion stabilization in δ -chymotrypsin–inhibitor complexes

The similarity in the pK_a values determined from K_i values (Figure 1b) and from hemiketal oxyanion formation (Table 2) suggests that protonation of the hemiketal oxyanion results in a 21.9-fold increase in K_i , which destabilizes the inhibitor complex

by 7.7 kJ·mol $^{-1}$. Protonation of the oxyanion should decrease the strength of each of its hydrogen bonds by 5.1–23.1 kJ·mol $^{-1}$ [16]. The contribution of multiple hydrogen bonds is expected to be at least additive [42], and possibly greater than this if they act as pre-aligned dipoles [43]. Therefore, as there are two hydrogen bonds to the oxyanion in chymotrypsin, then protonation of the oxyanion should decrease the strength of oxyanion binding by at least 10.2–46.2 kJ·mol $^{-1}$. The observed decrease of 7.7 kJ·mol $^{-1}$ is less than predicted, which can be explained if the low oxyanion pK_a value (Table 2) results from a low charge density on the oxyanion. From this decrease in binding energy, we estimate that hydrogen bonding in the oxyanion hole lowers the oxyanion pK_a by 1.3 pK_a units in chymotrypsin. Assuming that the hydrogen bonds from Gly-193 and Ser-195 are of the same strength, then each hydrogen bond will lower the oxyanion pK_a by 0.65 pK_a unit. Using site-directed mutagenesis to delete the side chain of Asn-155 of subtilisin, we estimated that a single hydrogen bond lowered the oxyanion pK_a by 1.09 pK_a units in a subtilisin–chloromethane-inhibitor derivative [16]. The larger decrease in pK_a with the oxyanion in the subtilisin–chloromethane-inhibitor adduct is most probably due to its higher pK_a (Table 2), giving it a higher charge density on its oxyanion. These results support our earlier conclusion [16,19,20,22,23,25] that most of the decrease in the oxyanion pK_a value is due to its interaction with the imidazolium cation of the active-site histidine residue, and that the main role of hydrogen bonding by the oxyanion is to provide localized solvation of the oxyanion.

There was no evidence that binding of the Z-Ala-Pro-Phe-glyoxal inhibitor was influenced by a pK_a of ~ 5.3 due to the formation of a hydrate oxyanion (structure 3 in Scheme 3). This suggests that the hydrate oxyanion is not stabilized significantly by hydrogen bonding, which would be expected if, as we suggest, it is not in the oxyanion hole. However, chymotrypsin lowers the hydrate oxyanion pK_a (structures 2 and 3 in Scheme 3) slightly more than it does the pK_a of the hemiketal oxyanion (structures 2 and 4 in Scheme 3) in the oxyanion hole (Table 3). This result can be explained by the fact that we would expect the hydrate oxyanion to be nearer the imidazolium ion of His-57, and so its pK_a would be decreased more effectively by its electrostatic interaction with the imidazolium ion. Glyoxal inhibitors of chymotrypsin and thiol proteases have been shown to be 3–10-fold more effective than the equivalent aldehyde inhibitors [7]. It has been suggested that the better binding by the glyoxal inhibitors is due to the hydrated β -aldehyde hydroxy groups forming additional hydrogen bonding interactions within the active site of thiol proteases [8,9]. The failure to detect better binding when the hydrate oxyanions are formed with a pK_a of ~ 5.3 suggests that such additional hydrogen bonding interactions do not have a significant effect on binding in the chymotrypsin–Z-Ala-Pro-Phe-glyoxal complex.

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