13C- and 1H-NMR studies of ionisations and hydrogen bonding in chymotrypsin-glyoxal inhibitor complexes*
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Running Title: NMR studies of chymotrypsin-glyoxal inhibitor complexes
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Z-Ala-Pro-Phe-glyoxal and Z-Ala-Ala-Phe-glyoxal have both been shown to be inhibitors of α-chymotrypsin with minimal K_i values of 19 nM and 344 nM respectively at neutral pHs. These K_i values increased at low and high pHs with pK_a values of ~4.0 and ~10.5 respectively. Using surface plasmon resonance we show that the apparent association rate constant for Z-Ala-Pro-Phe-glyoxal is much lower than the value expected for a diffusion controlled reaction. 13C-NMR has been used to show that at low pH the glyoxal keto carbon is sp3 hybridised with a chemical shift of ~100.7 p.p.m. and that the aldehyde carbon is hydrated with a chemical shift of ~91.6 p.p.m. The signal at ~100.7 p.p.m. is assigned to the hemiketal formed between the hydroxy-group of serine-195 and the keto carbon of the glyoxal. In a slow exchange process controlled by a pK_a of ~4.5 the aldehyde carbon dehydrates to give a signal at ~205.5 p.p.m. and the hemiketal forms an oxyanion at ~107.0 p.p.m. At higher pH re-hydration of the glyoxal aldehyde carbon, leads to the signal at 107 p.p.m. being replaced by a signal at 104 p.p.m. (pK_a ~9.2). On binding either Z-Ala-Pro-Phe-glyoxal or Z-Ala-Ala-Phe-glyoxal to α-chymotrypsin, at 4 and 25 °C 1H-NMR is used to show that the binding of these glyoxal inhibitors raises the pK_a of the imidazolium ion of histidine-57 to a value >11 at both 4 and 25°C. We discuss the mechanistic significance of these results and we propose that it is ligand binding which raises the pK_a of the imidazolium ring of histidine-57 allowing it to enhance the nucleophilicity of the hydroxy group of the active site serine-195 and lower the pK_a of the oxyanion forming a zwitterionic tetrahedral intermediate during catalysis.

Specific substrate derived glyoxal inhibitors have been shown to be potent inhibitors of the serine proteinases (1-4). Z-Ala-Pro-Phe-glyoxal2 is an extremely potent reversible inhibitor of δ-chymotrypsin with an apparent disassociation constant of 25 ± 8 nM at pH 7.0 (1).

The α-keto carbon of the glyoxal inhibitor is expected to occupy the same position as the carbonyl carbon of a substrate and it has been shown that it is bound as a tetrahedral adduct which should closely resemble the tetrahedral intermediate formed during substrate catalysis (1). Using 13C-NMR it has been shown that δ-chymotrypsin (1) and subtilisin (2) reduce the oxyanion pK_a by ~6 and ~8 pK_a units respectively. It has been estimated that hydrogen bonding in the oxyanion hole will only reduce the oxyanion pK_a by ~1.3 pK_a units (1). This is consistent with the fact that hydrogen bonding is expected to be effective in both water and in the oxyanion hole and so it should not reduce the oxyanion pK_a to a value lower than that expected in water. This has led to the conclusion that hydrogen bonding in the oxyanion hole only has a minor role in lowering the oxyanion pK_a (5-7). It has however been proposed that substrate binding raises the pK_a of the imidazolium ion of the active site histidine enabling it to promote ionisation of the active site serine hydroxyl enhancing its nucleophilicity for catalysis and to also reduce the oxyanion pK_a when a tetrahedral intermediate is formed (5). Binding by inhibitors such as glyoxals is expected.
to have a similar effect raising the pK_{a} of the imidazolium ion of the active site histidine which then reduces the oxyanion pK_{a}. However, there is no direct evidence that the pK_{a} of the imidazolium ion of histidine-57 is raised on binding glyoxal inhibitors. Therefore one of the aims of this work is to examine the ionisation state of histidine-57 in the presence of glyoxal inhibitors.

It has been shown that ¹H-NMR. can be used to observe the N^{\delta^{1}} and N^{\delta^{2}} protons of the active site histidine in chymotrypsin and subtilisin with or without ligands bound (8-10). Therefore in the present work we have used ¹H-NMR to observe the hydrogen bonded proton located between the carboxylate group of of aspartate-158 and N^{\delta^{1}} of histidine-57 in both free α–chymotrypsin and in α–chymotrypsin-glyoxal inhibitor complexes. We have shown that glyoxal inhibitors are bound at alkaline pHs under our NMR conditions ([E] ~ [I] ~1mM). This has allowed us to determine whether the pK_{a} of the active centre histidine residue is raised in α–chymotrypsin-glyoxal inhibitor complexes. We have also used ¹³C-NMR to examine ionisations within the Z-Ala-Pro-Phe-glyoxal / α–chymotrypsin complex. Using Z-Ala-Ala-Phe-glyoxal we have been able to assess how proline contributes to binding and whether it affects the observed protonic equilibria within the α–chymotrypsin/ glyoxal inhibitor complexes.

Experimental procedures

Materials - L-[1-¹³C]Phenylalanine (99 atom %) was obtained from Cambridge Isotope Laboratories, Inc. (50, Frontage Road, Andover, MA 01810-5413 USA) and from CDN Isotopes (88 Leacock Street, Pointe-Claire, Quebec). All other chemicals used were obtained from the Sigma-Aldrich Chemical Co., Gillingham, Dorset, U.K.

Inhibitor Synthesis - Z-Ala-Pro-Phe-glyoxal, Z-Ala-Pro-Phe-[2-¹³C]glyoxal, Z-Ala-Ala-Phe-glyoxal, Z-Ala-Ala-[1-¹³C]Phe-glyoxal and Z-Ala-Ala-Phe-[2-¹³C]glyoxal were synthesized using the methods described by Djurdjevic-Pahli et al., (1).

Enzyme solutions - α–chymotrypsin (crystallized and lyophilized) was obtained from Sigma-Aldrich Chemical Co. and the amount of fully active protein (69%) was determined as described by Finucane et al., (11).

Inhibition of α–chymotrypsin by Z-Ala-Pro-Phe-glyoxal and Z-Ala-Ala-Phe-glyoxal - The inhibition of the α–chymotrypsin catalysed hydrolysis of Succinyl-α-Ala-Pro-Phe-nitroanilide by Z-Ala-Pro-Phe-glyoxal and Z-Ala-Ala-Phe-glyoxal were studied at 25°C in 0.1M buffers containing 3.3% (v/v) dimethyl sulfoxide. The buffers used were potassium formate (pH 2.9-4.5), sodium acetate (3.8-5.6), potassium phosphate pH 6.2-8.2 and ~pH 12) and sodium carbonate (pH 9.7-11.0). K_i values were estimated when [So] << K_M. Therefore the equation for competitive inhibition (d[P]/dt=(k_{cat}[E]*[S])/(S)+K_i*[1+[I]/K_i) reduces to (d[P]/dt=(k_{cat}/K_M)*[E]*[S]*K_i/[I]+K_i).

Rearranging this equation and multiplying both sides by the inhibitor concentration gives ([I]*d[P]/dt/[E]/[S]=2(k_{cat}/K_M)*K_i/[I]/([I]+K_i).

Therefore a plot of [I]*d[P]/dt/[E]/[S] versus [I] gives a hyperbolic graph which can be analysed in the same way as Michaelis Menten data. Stock solutions of 25 mM substrate and 250 mM inhibitor were dissolved in dimethyl sulfoxide and quantified. For Z-Ala-Pro-Phe-glyoxal final concentrations of the enzyme, substrate and inhibitor were in the ranges 0.001-0.06 μM, 0.004-0.11 mM and 0.01-2 μM respectively.

Surface Plasma Resonance - Surface Plasma Resonance experiments were performed using a Biacore X instrument (Biacore, Uppsala, Sweden). α-Chymotrypsin was immobilised on a carboxymethylated sensor chip (CM5). The sensor carboxyl groups were activated by treatment with N-ethyl-N-(dimethylaminopropyl)-carbodiimide and N-hydroxysuccinimide. α–chymotrypsin (10 μg/ml) in 10 mM sodium acetate buffer at pH 5.5 was injected over a 7 minute time period at a flow rate of 5 μl/min. Non-reacted N-hydroxysuccinimide esters were deactivated by injecting with 1 M ethanolamine for 7 minutes at the same flow rate. Protein inhibitor interactions were studied at pH 7.23 using filtered and degassed 10mM phosphate buffer. The inhibitor was injected at varying concentrations (6.2 nM to 248 nM) over a 2 minute time period at a flow rate of 50 ul/min.

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. 10 mm-diameter sample tubes were used. The $^{13}$C-NMR spectral conditions for the samples of chymotrypsin inhibited by Z-Ala-Pro-$[2,13^\text{C}]$Phe-glyoxal or Z-Ala-Ala-$[2,13^\text{C}]$Phe-glyoxal at 11.75 T were: 32768 time-domain data points; spectral width 240 p.p.m.; acquisition time 0.541 s; 8.0 s relaxation delay time; 90° pulse angle; 200 transients were recorded per spectrum. Waltz-16 composite pulse $^1$H decoupling with a BLARH100 amplifier was used with 16 dB attenuation during the acquisition time and 34 dB attenuation during the relaxation delay to minimise dielectric heating but maintain the Nuclear Overhauser Effect. Spectra were transformed using an exponential weighting factor of 20 Hz. Samples of chymotrypsin inhibited by Z-Ala-Pro-$[1,13^\text{C}]$Phe-glyoxal were examined under the same conditions except that acquisition time was 0.135 s, the relaxation delay was 0.6 s and 2320 transients were recorded per spectrum. Spectra were transformed using an exponential weighting factor of 40 Hz.

$^1$H-NMR spectra were obtained at 500 MHz using 5 mm-diameter sample tubes. The $^1$H-NMR spectral conditions for the samples of chymotrypsin inhibited by Z-Ala-Pro-Phe-glyoxal or Z-Ala-Ala-Phe-glyoxal at 11.75 T were: 32768 time-domain data points; spectral width 40 p.p.m.; acquisition time 0.818 s; 1.0 s relaxation delay time; 90° pulse angle; 128 transients were recorded per spectrum. Water suppression was achieved using the Watergate W5 pulse sequence with gradients (12). Spectra were transformed using an exponential weighting factor of 50 Hz. $^{13}$C-NMR spectra confirming the formation of the $\alpha$-chymotrypsin inhibitor complex with either 1- or 2-$^{13}$C-enriched glyoxal inhibitors were obtained prior to $^1$H-NMR studies.

Both $^1$H and $^{13}$C chemical shifts are quoted relative to tetramethylsilane at 0.00 p.p.m. In aqueous solutions the chemical shift of the $\alpha$-carbon of glycine was used as a chemical reference as described previously (11). For non-aqueous solvents either 10% tetramethylsilane was used as an internal standard or an appropriate solvent signal was used as a secondary reference (13).

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

RESULTS

Inhibition of the $\alpha$-chymotrypsin catalysed hydrolysis of Succinyl-Ala-Ala-Ala-Pro-p-nitroanilide by Glyoxal inhibitors Z-Ala-Ala-Phe-glyoxal and Z-Ala-Pro-Phe-glyoxal - $K_i$ values were estimated at 25°C (Table 1) when [So] << $K_M$ and $d[\text{P}]/dt=(k_{\text{cat}}/K_M)*[\text{E}]*[\text{S}]*K_i/([I]+K_i)$.

The binding constants for Z-Ala-Ala-Phe-glyoxal increased ~46 fold from 0.34 ± 0.02 µM to 15.6 ± 0.8 mM at low pH and ~5.2 fold to 1.77 ± 0.31 µM at high pH according to a pK$_a$ of 4.32 ± 0.14 and 10.70 ± 0.44 respectively (Fig. 1a). The binding of Z-Ala-Ala-Phe-glyoxal to $\alpha$-chymotrypsin at pH 7 is ~10 x less effective than with Z-Ala-Pro-Phe-glyoxal binding to $\delta$-chymotrypsin at pH 7 (1). The binding constants for Z-Ala-Pro-Phe-glyoxal increased ~19 fold from 0.033 ± 0.002 µM to 0.63 ± 0.06 µM at low pH and ~9.1 fold to 0.30 ± 0.03 µM at high pH according to a pK$_a$ of 4.00 ± 0.21 and 10.41 ± 0.22 respectively (Fig. 1b). In our NMR experiments we use concentrations of enzyme and inhibitor of ~1 mM. Therefore the enzyme and inhibitor concentrations are ~3300 and ~560 fold greater than the $K_i$ values obtained with Z-Ala-Pro-Phe-glyoxal and Z-Ala-Ala-Phe-glyoxal respectively confirming that the inhibitor will be bound to the enzyme in our NMR experiments even at pHs 3 and 12.

The pK$_a$ values of 4.0-4.3 determined from the increase in $K_i$ values at low pH (Fig. 1) are similar to that assigned to protonation of the hemiketal oxyanion in the enzyme inhibitor complex (Structure E to B, Fig. 8). The pK$_a$ values determined from the pH dependence of $K_i$ or $K_d$ values will reflect ionisations within the enzyme-inhibitor complex (14). Therefore the pK$_a$ values of 4.0-4.3 observed with both inhibitors are assigned to hemiketal oxyanion formation in the enzyme inhibitor complex (Structure B to E, Fig. 8).

The ionisation of the isoleucine-16/aspartate-104 ion pair in free $\alpha$-chymotrypsin occurs with a pK$_a$ value of 8.8 and it causes a conformational change which reduces catalytic activity (15). The binding of ligands raises the pK$_a$
of the ion pair to ~10.5 (16) which is in good agreement with the pKₐ of 10.4-10.7 obtained from the pH dependence of the Kₐ values when either of the inhibitors are bound to α-chymotrypsin. Therefore pKₐ's of 10.4-10.7 are assigned to a conformational change which decreases the Kₐ values for inhibitor binding to α-chymotrypsin.

Examination of inhibitor binding using surface plasmon resonance - Surface plasmon resonance is widely used to determine binding constants and the on (kₐ) and off (kₖ) rate constants for ligand binding (17-19). Chymotrypsin was immobilised on the sensor chip by forming amide bonds between its free amino groups and the carboxyl groups of the sensor chip matrix as described in the Materials and Methods section.

If the association rate constant (kₐ) is diffusion controlled (~10⁸ M⁻¹ s⁻¹) then separate signals for the free and bound ligands will only be observed for tightly bound inhibitors with apparent Kₐ values of < ~1 μM (20). With Z-Ala-Ala-Phe-glyoxal the Kₐ values at low pH were > 10 μM. However, separate signals were observed for both free and bound inhibitor (Fig. 2B). Using surface plasmon resonance the rates of association (kₐ) and dissociation (kₖ) for Z-Ala-Pro-Phe-glyoxal were estimated to be 4.83 x 10⁵ M⁻¹ s⁻¹ and 9.30 x 10⁻³ s⁻¹ respectively at pH 7.23. Similar low rates of association were estimated at pH 4.28 from line broadening measurements (1). This explains why signal coalescence (of the free and bound ligand signals) and fast exchange broadening are not significant effects in the NMR spectra presented (Fig. 2), even when the apparent dissociation constants approach mM values (Fig. 1) at low and high pH values (20).

At ~pH 7 the dissociation constants for α-chymotrypsin and Z-Ala-Pro-Phe-glyoxal determined by inhibition kinetics and surface plasmon resonance were essentially the same (Table 1).

¹³C-NMR of the α-chymotrypsin inhibited by Z-Ala-Pro-[2-¹³C]Phe-glyoxal and Z-Ala-Pro-[1-¹³C]Phe-glyoxal - In our earlier studies (1) we studied the interaction of Z-Ala-Pro-[2-¹³C]Phe-glyoxal with δ-chymotrypsin from pH 3.3 to 6.9 at 25°C. δ-chymotrypsin is no longer commercially available and so to confirm that our earlier studies with δ-chymotrypsin are comparable to those we obtain with α-chymotrypsin in the present work we have repeated our earlier studies with Z-Ala-Pro-Phe-glyoxal using α-chymotrypsin instead of δ-chymotrypsin. We have also undertaken new studies at pHs 7-11.0 (Fig 2A). On adding a slight excess of Z-Ala-Pro-[2-¹³C]Phe-glyoxal to α-chymotrypsin a new signal at 100.7 p.p.m. was observed (Fig. 2A1). A small additional signal at 96.5 p.p.m. due to the excess free inhibitor was also observed (Fig. 2A1). On increasing the pH the chemical shift of the signal at 100.7 p.p.m. increased (Fig. 2A) to 104.3 p.p.m. at pH 11.1 p.p.m. (Fig. 2A11). A plot of chemical shift versus pH (Fig. 3) revealed that there is a biphasic increase in chemical shift as the pH is increased. Initially there was an increase from 100.68 ± 0.02 p.p.m. to 104.02 ± 0.02 p.p.m with a pKₐ of 5.23 ± 0.03 (Fig. 3). Then, there was a further small increase in chemical shift from 104.02 ± 0.02 to 104.26 ± 0.02 p.p.m. with a pKₐ of 8.83 ± 0.49 (Fig. 3). This pKₐ is similar to that attributed to the ionisation of the isoleucine-16/ aspartate-194 ion pair in the free enzyme which inactivates chymotrypsin when the carboxy-group of aspartate-194 moves and protrudes into the active site increasing Kₐ values. However, this pKₐ is usually raised when ligands bind or if the hydroxy-group of serine-195 is chemically modified (11).

Analysis of the pH dependent changes in dissociation constants shows that the efficiency of binding of Z-Ala-Pro-Phe-glyoxal and Z-Ala-Pro-Phe-glyoxal by α-chymotrypsin decreases with pKₐ's of 10.43 (Fig. 1b) and 10.67 ± 0.24 (Fig. 1a) respectively. This shows that the ionisation of the isoleucine-16/ aspartate-194 ion pair must have a pKₐ ≥ 10.43-10.67. Therefore the pKₐ of ~9 cannot be assigned to ionisation of the isoleucine-16/ aspartate-194 ion pair. The pKₐ of 5.23 obtained with α-chymotrypsin at 25 °C is identical to the value obtained for δ-chymotrypsin (Table 2) and it is assigned as in earlier studies (1) to the ionization of the hydrated aldehyde hydroxy groups of the inhibitor (Structures B and C in Fig. 8).

Most ¹H-NMR studies of the hydrogen bonded protons of chymotrypsin have been undertaken at 4 °C to minimise exchange broadening. As we have undertaken similar ¹H-NMR studies with Z-Ala-Pro-[2-¹³C]Phe-glyoxal
and α–chymotrypsin we have also undertaken low temperature $^{13}$C-NMR studies to determine how temperature affects the pKa values determined. With Z-Ala-Pro-[2-$^{13}$C]Phe-glyoxal and α–chymotrypsin lowering the temperature to 4 °C produced a small 0.3 pH unit increase in the pKa from 5.23 to 5.71 and a small 0.2 pH unit decrease in the second pKa from 8.83 to 8.59 (Table 2). This suggests that both ionising groups have low enthalpies of ionisation.

The signal at ~100.7 to ~104 p.p.m. in the Z-Ala-Pro-[2-$^{13}$C]Phe-glyoxal/δ–chymotrypsin complex has been has been shown to be in slow exchange with a signal at ~107 p.p.m. which is formed with a pKa ~4.5 (1). We have obtained similar results were obtained with the Z-Ala-Pro-[2-$^{13}$C]Phe-glyoxal/α–chymotrypsin complex (Fig. 2A). However, on extending our observations to alkaline pH values it was found that the intensity of the signal at ~107 p.p.m. had a bell shaped pH dependence (Fig. 4a) increasing with a pK$_a$ of 4.45 ± 0.16 to a maximum intensity at ~pH 5.7 (Fig. 2A5) and decreasing in intensity with a pK$_a$ of 7.81 ± 0.15 at 25 °C (Fig. 4a). Similar results were obtained at 4 °C (Fig. 4b). However, while there was only a small increase of 0.4 in pK$_a$ there was a larger increase of 1.21 in pK$_a$ suggesting that pK$_a$ may be due to cationic acid such as an amino group or imidazolium group. The hydration of aldehyde carbonyl groups is subject to general acid-base catalysis (21). Therefore hydration is usually minimal at neutral pHs but increases at higher or lower pHs while aldehyde concentrations decrease at high or low pH values. The intensities of the signals at ~107 p.p.m. showed the expected pH dependence, decreasing in intensity at high or low pHs. This adds additional support to our earlier assignment of the signals at ~107 p.p.m. to structure E in Fig. 8. This scheme has been modified from the scheme presented in earlier work(1) to allow for the re-hydration (Structures E to C in Fig. 8) of the aldehyde carbonyl (Structure E in Fig. 8) at high pH.

The signal ~104 p.p.m. reached a maximum intensity at alkaline pHs (Fig. 2A) with a pK$_a$ of 9.2 ± 0.1 (Fig. 5a). This change in signal intensity is assigned to the hydration of the aldehyde carbon at alkaline pHs (Structures E to C in Fig. 8).

When α–chymotrypsin was inhibited by Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal a new signal was observed which titrated from 91.6 ± 0.15 p.p.m at low pH to 97.55 ± 0.11 p.p.m. with increasing pH according to a pK$_a$ of 5.26 ± 0.06. This titration behaviour is essentially the same as that observed using δ–chymotrypsin. The intensity of the signal at ~205 p.p.m. increased with a pK$_a$ of 3.83 ± 0.21 and decreased with a pK$_a$ of 8.29 ± 0.21. A similar bell shaped pH dependence was seen for the signal at 107 p.p.m. which is consistent with the formation and loss of species E in Fig. 8. However while the signal at 104 p.p.m due to the quaternary carbon (Structure C in Fig. 8) was detected at high pH, the signal at 97.55 p.p.m. was not detected at high pH, presumably due to the larger linewidth of the aldehyde carbon.

$^{13}$C-NMR of the α–chymotrypsin inhibited by Z-Ala-Ala-[1-$^{13}$C]Phe-glyoxal and Z-Ala-Ala-[2-$^{13}$C]Phe-glyoxal - Similar results were obtained at 25 °C using Z-Ala-Ala-[2-$^{13}$C]Phe-glyoxal and α–chymotrypsin (Fig. 2B) except that for the signal titrating from 100.79 to 104.00 p.p.m. to 104.32 there was a small increase of ~0.3 pH units in the first pK$_a$ to 5.52 and a larger ~0.9 pH unit decrease in the second pK$_a$ to 7.89 at 25 °C (Table 2). The intensity of the signal at 107.8 p.p.m., had a similar bell shaped pH dependence and again at 25 °C there was a small increase in the first pK$_a$ to 4.63 but there was a larger ~1 p.p.m. increase in the second pK$_a$ to 8.87 (Table 2b). The intensity of the signal at 104.3 p.p.m. again reached a maximum intensity at alkaline pHs (Fig. 2B) with a pK$_a$ of 8.92 ± 0.1 (Fig. 5b).

With Z-Ala-Ala-[1-$^{13}$C]Phe-glyoxal at 25 °C the intensity of the signal at 205.7 p.p.m. showed a bell shaped pH dependence (Table 3) similar to that seen for the signal at ~107 p.p.m. The signal due to the hydrated aldehyde carbon of the Z-Ala-Ala-[1-$^{13}$C]Phe-glyoxal inhibitor bound to α–chymotrypsin titrated with increasing pH from 90.73 ± 0.04 to 96.69 ± 0.03 according to a pK$_a$ = 5.62 ± 0.02 which is similar to the titration results obtained with Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal and a δ–chymotrypsin (Table 2). But as we observed with the Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal inhibitor complex and α–chymotrypsin the signal at 96.69 p.p.m. (97.55 p.p.m. with Z-Ala-Pro-[2-$^{13}$C]Phe-glyoxal) did not re-appear at high pH as expected (i.e. signal at 97.4 in Structures C and D in Fig. 8).

The formation of the signal at ~107 p.p.m. and 205.2 p.p.m. (Structure 1E in Fig. 8) occurred...
with a pKᵢ values of 4.5-4.9 and the loss of these signals occurred with pKᵢ values of 7.8 and 10.0 respectively. The formation of the signal at 104 p.p.m. occurred with a pKᵢ of 8.9-9.2. Therefore the one step conversion of E to C in Fig. 8 may be a simplification.

\textit{H-NMR of the hydrogen bonded protons of α-chymotrypsin and of its complex with Z-Ala-Pro-Phe-glyoxal} - The hydrogen bonded proton at ~18 p.p.m. in unligated α-chymotrypsin has been assigned to the N⁶¹ proton of the imidazolium ion of histidine-57 which is hydrogen bonded to aspartate-32 (8,10,22). At 4 °C this proton titrated from 18.0 ± 0.1 at low pH to 14.7 ± 0.1 at high pH with a pKᵢ of 6.7 ± 0.1 at 4 °C. This value for α-chymotrypsin is lower than the value of 7.5 obtained using δ-chymotrypsin at 3 °C (10). However, it is in good agreement with the value of 6.56 ± 0.13 obtained the pH dependence of 1/Kᵢ for Z-Ala-Ala-Phe-glyoxal binding to α-chymotrypsin at 25°C (data not shown). This confirms that under our experimental conditions the active site histidine-57 of free α-chymotrypsin has a pKᵢ of ~6.7. A signal at 13.0 p.p.m. was detected at low pH which could not be observed at pHs > 5.1. This signal has been observed in chymotrypsinogen and has been assigned to the N⁵⁷ proton of histidine-57 (23). It has also been observed before in α-chymotrypsin at low pH (24,25) and in an α-chymotrypsin inhibitor complex with a trifluoromethylketone inhibitor (25). The signal at ~13.0 p.p.m. has been assigned to the N⁵⁷ proton of the imidazolium ring of histidine-57 of α-chymotrypsin (24).

When Z-Ala-Pro-Phe-glyoxal was added to α-chymotrypsin at pH 3.42 signals at 18.4 and 12.8 p.p.m. were observed at 4 °C (Fig. 6A1). In a complex between Ac-Leu-Phetrifluoromethylketone similar signals were observed at 18.7 and 12.8 p.p.m. as well as signals at 18.0 and 13.1 p.p.m. due to free α-chymotrypsin (25). The signal at 18.7 p.p.m. was assigned to the N⁶¹ proton of histidine-57 in the α-chymotrypsin / Ac-Leu-Phetrifluoromethylketone inhibitor complex but the signal at ~13 p.p.m. was not assigned (25). We assign the signals at 18.4 and 12.8 p.p.m. at 4 °C in the Z-Ala-Pro-Phe-glyoxal/α-chymotrypsin complex to the N⁶¹ and N⁵⁷ protons respectively of the imidazolium ring of histidine-57. The signals at 18.7 p.p.m. and 13.0 p.p.m. that were observed at a similar pH but at 25 °C (Fig. 6B1) were assigned in the same way. These signals confirm that the imidazolium ion of histidine-57 is present at low pH in the Z-Ala-Pro-Phe-glyoxal/α-chymotrypsin complex at 4 °C (Fig. 6A1) and 25 °C (Fig. 6B1). These signals are also present at high pHs with slightly different chemical shifts of 13.3-13.6 p.p.m. and 17.2-17.5 p.p.m. at both 4 °C (Fig. 6A9-11) and 25 °C (Fig. 6B8-11). This demonstrates that the pKᵢ of the imidazolium ion has been raised to a value >11 in the Z-Ala-Pro-Phe-glyoxal/α-chymotrypsin complex.

At 4°C the NMR signals broaden and disappear as the pH is increased to ~9.4 (Fig. 6A2-8) but re-appear along with additional signals at pHs 10-11 (Fig. 6A9-11). At 25 °C the signals persist over much larger pH ranges (Fig. 6B1-11). Robillard and Schulman reported (10) that in chymotrypsin chloromethylketone inhibitor adducts, oxyanion formation could be detected by a small titration shift from 17.25 to ~16.3 p.p.m. We have examined the signals at ~13 and ~18 p.p.m. for similar small pH dependent changes in chemical shift (Fig. 6) which we hoped could be attributed to ionisations within the enzyme-inhibitor complex. At 25 °C with Z-Ala-Pro-Phe-glyoxal the signals at ~13 p.p.m. and at ~18 p.p.m. were detected over a wider range of pH values (Fig. 6B) than at 4 °C (Fig. 6A). The signal at ~18 p.p.m. (Fig. 6B) decreased in chemical shift from 18.72 ± 0.04 to 18.32 ± 0.02 and to 17.40 ± 0.01 p.p.m. as the pH was raised (Fig. 7) and these decreases depended on pKᵢ values of 4.17 ± 0.34 and 8.18 ± 0.19 respectively which appear to reflect the exchange processes converting B to E to C in Fig. 8. Therefore it appears that the N⁶¹ proton of histidine-57 is being affected by the formation of both the hemiketal oxyanion (Structure E in Fig. 8) and the hemiacetal oxyanion (Structure C in Fig. 8). The signal at ~13 p.p.m. showed smaller increases in chemical shift as the pH increased (Fig. 6) which were too small to be reliably analysed for pKᵢ values.

At 4°C with Z-Ala-Pro-Phe-glyoxal a signal at 14.7 p.p.m. appeared at pHs 10.43 (Fig. 6A11) and 11.03 (Fig. 6A12) due to the N⁶¹ proton of the imidazolium ring of histidine-57 suggesting that some free α-chymotrypsin had been formed at the
highest pH values due to alkali catalysed breakdown of the inhibitor (1).

An additional signal at 16.4-16.6 p.p.m. was also detected at high pHs in both inhibitor complexes at 4 °C and 25 °C. A similar signal has been observed in chloromethylketone derivatives of α-chymotrypsin and it was in slow exchange with a signal at 17.3 p.p.m. at 3 °C but they were in fast exchange at 16 °C and titrated with a pKₐ of 8.4 (10). It was suggested that this signal was due to the imidazole N° proton and the pKₐ resulted from the conformational change resulting from the pH dependent disassociation of the tetrahedral adduct. Likewise formation of this signal in our experiments could reflect the conformational change which makes inhibitor binding weaker at high pH.

'¹H-NMR of the hydrogen bonded protons of α-chymotrypsin and of its complex with Z-Ala-Ala-Phe-glyoxal - When Z-Ala-Ala-Phe-glyoxal was incubated with α-chymotrypsin at 25 °C signals at 18.7 and 13.4 p.p.m. were detected at low pH and signals at 17.4, 16.6 and 13.5 p.p.m. were detected at high pHs (Fig. 6C) as was observed when α-chymotrypsin was incubated with Z-Ala-Pro-Phe-glyoxal (Fig. 6B). All these signals (Fig. 6C) underwent similar pH dependent changes to those observed with Z-Ala-Pro-Phe-glyoxal (Fig. 6B). The chemical shift of the signal at ~18 p.p.m. reflected (Table 4) both the formation of both the hemiketal oxyanion (Structure E in Fig. 8) and the hemiacetal oxyanion (Structure C in Fig. 8).

DISCUSSION

Z-Ala-Pro-Phe-glyoxal can exist in both cis and trans forms with the trans form (91%) predominating over the cis form (9%) in dimethyl sulfoxide (1). The carbon atoms can have different chemical shifts in the cis and trans forms. Therefore compounds like Z-Ala-Pro-Phe-glyoxal containing proline may give rise to up to twice as many ¹³C-NMR signals as compounds such as Z-Ala-Ala-Phe-glyoxal which do not contain proline. Careful examination of our spectra (e.g., Fig. 2) of α-chymotrypsin bound to Z-Ala-Pro[2-¹³C]Phe-glyoxal and Z-Ala-Ala-Phe-glyoxal gave no evidence of additional signals due to a mixture of cis/trans isomers. Therefore we conclude that Z-Ala-Pro-Phe-glyoxal bound predominantly in one form. Since chymotrypsin is specific for the trans isomer of substrates containing X-Ala-Pro-Phe-p-nitroanilide (26) then we propose that the Z-Ala-Pro-Phe-glyoxal inhibitor is bound in the trans form. Substitution of the alanine residue in the S₂ subsite with proline, led to a 12 fold improvement in binding. This is consistent with the observation that replacing alanine residues in the S₂ subsite with proline residues enhances substrate catalysis (27).

Surface plasmon resonance studies have shown that the apparent rate of association of glyoxal inhibitors with chymotrypsin is significantly slower (4.8 x 10⁸ M⁻¹·s⁻¹) than the diffusion controlled 10⁸ M⁻¹·s⁻¹) limit. This is because the kₐ is a two step process consisting of a diffusion controlled 2nd order association followed by a 1st order rate limiting step leading to the reversible formation of a tetrahedral adduct (Structures B to E in Fig. 8). This has greatly facilitated our ¹³C-NMR studies as there is no fast exchange broadening of the NMR signals.

At 25 °C the signal at ~107 p.p.m in the α-chymotrypsin/Z-Ala-Pro-Phe-glyoxal complex, assigned to the hemiketal oxyanion (Structure E in Fig. 8) is lost in slow exchange processes at low and high pH with pKₐ values of 4.5 and 7.8 respectively (Table 3). At low pH this loss correlates with the increase in the dissociation constant for the inhibitor (Table 1). This led to the suggestion that oxyanion formation promotes inhibitor binding. However, our current studies show that at alkaline pHs the dissociation constants for inhibitor binding to α-chymotrypsin increase with a pKₐ of ~10.7 with Z-Ala-Ala-Phe-glyoxal and with a pKₐ of 10.4 with Z-Ala-Pro-Phe-glyoxal (Fig. 1). The decrease in binding with these pKₐ values is thought to reflect the conformational change associated with the ionisation of the isoleucine-16-aspartate-194 ion pair. Inhibitor binding is expected to increase the pKₐ of the ion pair from 8.8 in free α-chymotrypsin to a value of ~10.5 in α-chymotrypsin-glyoxal inhibitor complexes. The fact that binding is apparently decreased by protonation of the hemiketal oxyanion but not by formation of the hemiacetal oxyanion (Fig. 8 E or B to C to D) suggests that the hemiacetal oxyanion...
does not make a significant contributions to inhibitor binding.

The monophasic titration shift of ~1 p.p.m. for the N^δ1 proton of histidine-57 in chloromethylketone derivatives of chymotrypsin has been attributed to oxygen ion formation (10). Therefore the larger 1.4 p.p.m. biphasic titration shift of this signal at ~18 p.p.m. in the glyoxal inhibitor complexes is expected as two oxygen atom interactions are formed in these inhibitor complexes (Fig. 8). In the chloromethylketone inhibitor complexes the oxygen atom is 5.7 Å from N^δ1 of histidine-57 (28). Using X-ray crystallographic data from Ac-Leu-Phe-CF₃ (29) the equivalent hemiketal oxygen atom in the glyoxal inhibitor complexes is estimated to be 7.4 from N^δ1 of histidine-57. This shows that the chemical shift of the N^δ1 proton of histidine-57 is affected by electrostatic interactions with groups up to at least ~7.5 Å.

Several studies have provided evidence for a low barrier hydrogen bond being formed when specific trifluoromethyl ketone inhibitors bind to chymotrypsin (30,31) raising the histidine pK_a to 11-12 (32). In this work with α-chymotrypsin and in earlier work with both δ-chymotrypsin (1) and subtilisin (2) we have shown that specific substrate derived glyoxal inhibitors form tetrahedral adducts analogous to the tetrahedral intermediate formed during catalysis and that the oxygen ion pK_a is reduced by 6-8 pK_a units. It has been suggested that the primary factor in reducing the pK_a of the oxygen ion is its electrostatic interaction with the imidazolium ion of histidine-57 (5,7,33,34). For these interactions to be effective the imidazolium ion pK_a must be similar (pK_a ~15) to that of the oxygen ion and serine hydroxyl group in the absence of this interaction (31,35). Our results show that the active site histidine residue is fully protonated up to pH 11 and so when the glyoxal inhibitor is bound the imidazolium ion of the active site must have a pK_a > 11. This shows that inhibitor binding raises the pK_a of the active site histidine residue. Therefore substrate binding should also raise the pK_a of histidine-57 enabling it to act as an efficient general base catalyst for deprotonation of the hydroxyl-group of the active site serine residue.

This will greatly enhance the nucleophilicity of the active site serine hydroxyl group, promoting tetrahedral intermediate formation and so promoting catalysis.

Raising the pK_a of the active site histidine residue by ligand binding will also allow its pK_a to approach or exceed that of conjugate acid of the oxygen ion and it will permit optimal electrostatic interaction between the imidazolium ion of histidine-57 and the oxygen ion as well as optimal hydrogen bonding between the imidazole group and the conjugate acid of the oxygen ion. These interactions should lower the oxygen ion pK_a and raise the pK_a of the imidazolium ion, resulting in the formation of a zwitterionic tetrahedral adduct with glyoxal inhibitors and zwitterionic tetrahedral intermediates during catalysis. Similar increases in the basicity of the active site histidine have also been observed when peptidyl trifluoromethyl ketones (25,32) or peptidyl boronic acid inhibitors (36) form negatively charged tetrahedral adducts with chymotrypsin. The pK_a of the active site histidine residue is also raised when subtilisin is covalently modified to give the monoisopropylphosphoryl enzyme (37). The pK_a shifts of monoisopropylphosphoryl chymotrypsin and subtilisin could be predicted if a dielectric constant of ~4 was used (38).

It has been suggested that the elevation of the histidine-57 pK_a on ligand binding is due to the decrease in the effective dielectric constant between the carboxylate group of aspartate-102 and the imidazolium ion of histidine-57 which enhances the electrostatic interaction between them (5). Mutations converting aspartate-102 into the neutral residues alanine (39) and asparagine (40,41) inactivated the enzyme while converting it to a negatively charged cysteine residue only led to a small decrease in catalytic efficiency (42). This supports the suggestion (5) that the increased electrostatic interaction between aspartate-102 and histidine-57 on binding ligands is an important factor in raising the pK_a of histidine-57 allowing it to enhance the nucleophilicity of the hydroxyl of serine-195 and to reduce the pK_a of the oxygen ion.
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**FOOTNOTES**

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2The Abbreviation used is: Z, benzyloxycarbonyl.

**FIGURE LEGENDS**

Fig. 1. **Effect of pH on the disassociation constants for the α–chymotrypsin inhibitor complexes formed with Z-Ala-Pro-Phe-glyoxal and Z-Ala-Ala-Phe-glyoxal at 25 °C.**

The experimental details are described in the Materials and methods section. The continuous lines were calculated using eqn. (1)

\[
K_{d(o)} = \frac{K_{d1}[H]^2 + K_{d2}K_{d3}[H] + K_{d3}K_{d4}K_{d5}}{([H]^2 + K_{d1}[H] + K_{d3}K_{d5})}
\]

The fitted parameters were: (a) Z-Ala-Ala-Phe-glyoxal: \(K_{d1} = 15.59 \pm 0.82 \mu M\), \(K_{d2} = 0.344 \pm 0.02 \mu M\), \(K_{d3} = 1.77 \pm 0.34 \mu M\), \(pK_{a1} = 4.32 \pm 0.14\) and \(pK_{a2} = 10.71 \pm 0.44\); (b) Z-Ala-Pro-Phe-glyoxal: \(K_{d1} = 0.678 \pm 0.110 \mu M\), \(K_{d2} = 0.033 \pm 0.0025 \mu M\), \(K_{d3} = 0.341 \pm 0.064 \mu M\), \(pK_{a1} = 3.95 \pm 0.30\) and \(pK_{a2} = 10.52 \pm 0.37\).

Fig. 2. **Effect of pH on the \(^{13}\)C-NMR signals from Z-Ala-Pro-[2-\(^{13}\)C]Phe-glyoxal and Z-Ala-Ala-[2-\(^{13}\)C]Phe-glyoxal in the presence of α–chymotrypsin.**

Acquisition and processing parameters were as described in the Materials and methods section. A: Sample (1) consisted of 2.25 ml of 1.85 mM Z-Ala-Pro-[2-\(^{13}\)C]Phe-glyoxal containing 1.72 mM α–chymotrypsin at pH 3.39. pHs were then changed as shown. In spectra (2), (3), (4), (5) and (6) the volumes of 1 M KOH containing 10% (v/v) \(^3\)H\(_2\)O added were 0.024, 0.01, 0.01, 0.01 and 0.008 ml respectively. Sample 7 was a new sample which consisted of 2.32 ml of 1.60 mM Z-Ala-Pro-[2-\(^{13}\)C]Phe-glyoxal containing 1.68 mM α–chymotrypsin at pH 7.54. In spectra (8), (9), (10) and (11) the volumes of 1 M KOH containing 10% (v/v) \(^3\)H\(_2\)O added were 0.016, 0.02, 0.024 and 0.012 ml respectively.

B: Sample (1) consisted of 2.96 ml of 1.36 mM Z-Ala-Pro-[2-\(^{13}\)C]Phe-glyoxal containing 1.40 mM α–chymotrypsin at pH 3.14. pHs were then changed as shown. In spectra (2), (3), (4), (5), (6) and (7) the volumes of 1 M KOH containing 10% (v/v) \(^3\)H\(_2\)O added were 0.060, 0.025, 0.030, 0.040, 0.020 and 0.010 ml respectively. Sample 8 was a new sample which consisted of 2.98 ml of 1.35 mM Z-Ala-Pro-[2-\(^{13}\)C]Phe-glyoxal containing 1.39 mM α–chymotrypsin at pH 8.53. In spectra (9), (10) and (11) the volumes of 1 M KOH containing 10% (v/v) \(^3\)H\(_2\)O added were 0.020, 0.010 and 0.010 ml respectively.

All samples were at 25 °C and they contained 10 mM potassium phosphate to help stabilize pH values and 10% (v/v) \(^2\)H\(_2\)O to maintain a deuterium lock signal. The glyoxal inhibitors were dissolved in \(d_6\)-DMSO and so all samples contained small amounts (0.6-2% (v/v)) of \(d_6\)-DMSO.

Fig. 3. **Effect of pH on the chemical shift of the \(^{13}\)C-enriched carbon of the Z-Ala-Pro-[2-\(^{13}\)C]Phe-glyoxal–α–chymotrypsin inhibitor complex.**

Experimental conditions are given in Fig. 2.

\[
\delta_{(o)} = \frac{\delta_1[H]^2 + \delta_2K_{a1}[H] + \delta_3K_{a3}K_{a2}}{([H]^2 + K_{a1}[H] + K_{a3}K_{a5})}
\]

The continuous line was calculated using equation (2) and the fitted parameters \(pK_{a1} = 5.23 \pm 0.04\), \(pK_{a2} = 8.83 \pm 0.49\), \(\delta_1 = 100.68 \pm 0.02\), \(\delta_2 = 104.02 \pm 0.02\) and \(\delta_3 = 104.26 \pm 0.02\).

Fig. 4. **Effect of pH on the intensity of the \(^{13}\)C-NMR signal at ~107 p.p.m. in the Z-Ala-Pro-[2-\(^{13}\)C]Phe-glyoxal–α–chymotrypsin complex at 25 °C and 4 °C.**
Experimental conditions are given in Fig. 2. 
\[ I_{\text{obs}} = \frac{I_{\text{max}}}{(1 + [H]/K_{a1} + K_{a2}/[H])} \]  
(3)
The continuous lines were calculated using equation (3). The fitted parameters were (a) at 25°C: pK\textsubscript{a1} = 4.45 ± 0.16, pK\textsubscript{a2} = 7.81 ± 0.15 and I\textsubscript{max} = 4.81 ± 0.35; (b) at 4°C: pK\textsubscript{a1} = 4.86 ± 0.14, pK\textsubscript{a2} = 9.02 ± 0.17 and I\textsubscript{max} = 3.88 ± 0.35

Fig. 5. Effect of pH on the intensity of the \textsuperscript{13}C-NMR signal at ~104 p.p.m. in the Z-Ala-Pro-[2-\textsuperscript{13}C]Phe-glyoxal-α-chymotrypsin complex at 25°C and 4°C.
Experimental conditions are given in Fig. 2. 
\[ I_{\text{obs}} = \frac{I_{\text{max}}}{(1 + [H]/K_{a1})} \]  
(4)
The continuous lines were calculated using equation (4). The fitted parameters were (a) at 25°C: pK\textsubscript{a1} = 9.19 ± 0.08 and I\textsubscript{max} = 5.64 ± 0.25; (b) at 4°C: pK\textsubscript{a1} = 8.92 ± 0.13 and I\textsubscript{max} = 5.55 ± 0.32.

Fig. 6. Effect of pH on the \textsuperscript{1}H-NMR signals from Z-Ala-Pro-[2-\textsuperscript{13}C]Phe-glyoxal in the presence of α-chymotrypsin at 4°C and 25°C.

Effect of pH on the \textsuperscript{13}C-NMR signals from Z-Ala-Pro-[2-\textsuperscript{13}C]Phe-glyoxal and Z-Ala-Ala-[2-\textsuperscript{13}C]Phe-glyoxal in the presence of α-chymotrypsin

Acquisition and processing parameters were as described in the Materials and methods section. A: Sample (1) consisted of 0.6 ml of 1.78 mM Z-Ala-Pro-[2-\textsuperscript{13}C]Phe-glyoxal containing 1.75 mM α-chymotrypsin at pH 3.42. pHs were then changed as shown. In spectra (2), (3), (4) and (11) the volumes of 1 M KOH containing 10\% (\textit{v/v}) \textsuperscript{2}H\textsubscript{2}O added were 7, 2, 1 and 10 \textmu l respectively. In spectra (5), (6), (7), (8), (9) and (10) the volumes of 0.1 M KOH containing 10\% (\textit{v/v}) \textsuperscript{2}H\textsubscript{2}O added were 16, 20, 10, 23, 25 and 50 \textmu l respectively.

B: Sample (1) consisted of 0.6 ml of 1.73 mM Z-Ala-Pro-[2-\textsuperscript{13}C]Phe-glyoxal containing 1.61 mM α-chymotrypsin at pH 3.31. pHs were then changed as shown. In spectra (2), (3), (4), (5), (6), (7), (8), (9), (10) and (11) the volumes of 1 M KOH containing 10\% (\textit{v/v}) \textsuperscript{2}H\textsubscript{2}O added were 10, 4, 1.8, 4.2, 4.2, 4.2, 6.6, 3.3, 3.3 and 4.2 \textmu l respectively.

C: Sample (1) consisted of 0.50 ml of 1.48 mM Z-Ala-Ala-[1-\textsuperscript{13}C]Phe-glyoxal containing 1.38 mM α-chymotrypsin at pH 4.06. pHs were then changed as shown. In spectra (2), (3), (4), (5), (6), (7), (8), (9), (10) and (11) the volumes of 1 M KOH containing 10\% (\textit{v/v}) \textsuperscript{2}H\textsubscript{2}O added were 6, 2, 1, 4, 1, 4, 2, 2, 2 and 2 \textmu l respectively.

All samples were at 25°C and they contained 10 mM potassium phosphate to help stabilize pH values and 10\% (\textit{v/v}) \textsuperscript{2}H\textsubscript{2}O to maintain a deuterium lock signal. The glyoxal inhibitors were dissolved in d\textsubscript{6}-DMSO and so all samples contained small amounts (0.7-1.3\% (\textit{v/v})) of d\textsubscript{6}-DMSO.

Fig. 7. Effect of pH on the chemical shift of the hydrogen bonded proton at ~18 p.p.m. in the Z-Ala-Pro-Phe-glyoxal-α-chymotrypsin inhibitor complex.
Experimental conditions are given in Fig. 6. 
\[ \delta_{\text{obs}} = \frac{[H]^2 + \delta_{\text{K}}[H] + \delta_{\text{K}}K_{\text{a}}K_{\text{a}}}{([H]^2 + K_{\text{a}}[H] + K_{\text{a}}K_{\text{a}})} \]  
(2)
The continuous line was calculated using equation (2) and the fitted parameters pK\textsubscript{a1} = 4.17 ± 0.34, pK\textsubscript{a2} = 8.18 ± 0.19, \delta_{\text{1}} = 18.72 ± 0.04, \delta_{\text{2}} = 18.32 ± 0.02 and \delta_{\text{3}} = 17.40 ± 0.01.

Fig. 8. Structures and chemical shifts of the α-chymotrypsin-glyoxal-inhibitor adducts.
TABLE 1
Disassociation constants for Z-Ala-Pro-Phe-glyoxal bound to α- and δ-chymotrypsin at 25°C

| pH  | Ki (µM) | Enzyme          | Method         | Reference         |
|-----|--------|-----------------|----------------|-------------------|
| 7.0 | 0.025  | δ-chymotrypsin  | Inhibition kinetics | Djurdjevic-Pahl(1) |
| 7.23| 0.019  | α-chymotrypsin  | Surface Plasmon Resonance | Present work |
| 7.17| 0.024  | α-chymotrypsin  | Inhibition kinetics | Present work |

TABLE 2
Titration constants obtained from pH dependent changes in the chemical shifts of the 13C-enriched signals in glyoxal inhibitor complexes formed with α- and δ-chymotrypsin

| Chymotrypsin | Inhibitor         | δ_1  | δ_2  | δ_3  | δ_2−δ_1 | pK_1 | pK_2 | pK_2 | °C  |
|--------------|-------------------|------|------|------|----------|------|------|------|-----|
| δ            | Z-Ala-Pro-[2-13C]Phe-glyoxal<sup>a</sup> | 100.70 | 103.94 | 3.24 | 5.23      | ND   | ND   | 25   |
| α            | Z-Ala-Pro-[2-13C]Phe-glyoxal<sup>b</sup> | 100.68 | 104.02 | 104.26 | 3.34 | 5.23 | 0.24 | 8.83 | 25  |
| α            | Z-Ala-Pro-[2-13C]Phe-glyoxal<sup>b</sup> | 100.83 | 103.91 | 104.28 | 3.24 | 5.71 | 0.37 | 8.59 | 4   |
| α            | Z-Ala-Pro-[2-13C]Phe-glyoxal<sup>b</sup> | 100.79 | 104.00 | 104.32 | 3.21 | 5.52 | 0.32 | 7.89 | 25  |
| α            | Z-Ala-Pro-[2-13C]Phe-glyoxal<sup>b</sup> | 104.02 | 104.26 | 0.24 | 8.83      | ND   | ND   | 25   |
| α            | Z-Ala-Pro-[2-13C]Phe-glyoxal<sup>b</sup> | 103.91 | 104.28 | 0.37 | 8.59      |      |      | 4    |
| δ            | Z-Ala-Pro-[1-13C]Phe-glyoxal<sup>a</sup> | 91.37 | 97.36 | 5.99 | 5.29      |      |      | 25   |
| δ            | Z-Ala-Pro-[1-13C]Phe-glyoxal<sup>b</sup> | 91.60 | 97.55 | 5.95 | 5.26      |      |      | 25   |
| α            | Z-Ala-Pro-[1-13C]Phe-glyoxal<sup>b</sup> | 90.73 | 96.69 | 5.96 | 5.62      |      |      | 25   |
| α            | Z-Ala-Pro-[2-13C]Phe-glyoxal<sup>b</sup> | 106.60 | 107.81 | 1.21 | 4.41      |      |      | 4    |

<sup>a</sup>Djurdjevic-Pahl, 2002, (2)
<sup>b</sup>Present work
**TABLE 3**
Titration constants obtained from pH dependent changes in the intensities of the $^{13}$C-enriched signals in glyoxal inhibitor complexes formed with $\alpha$-chymotrypsin

| Inhibitor | p.p.m. | $pK_1$ | $pK_2$ | °C |
|-----------|--------|--------|--------|----|
| Z-Ala-Pro-[$2^{-13}$C]Phe-glyoxal | 107.8  | 4.86   | 9.02   | 4  |
| Z-Ala-Pro-[$2^{-13}$C]Phe-glyoxal | 107.8  | 4.45   | 7.81   | 25 |
| Z-Ala-Ala-[$2^{-13}$C]Phe-glyoxal | 107.8  | 4.63   | 8.87   | 25 |
| Z-Ala-Pro-[$1^{-13}$C]Phe-glyoxal | 205.7  | 3.83   | 8.29   | 25 |
| Z-Ala-Ala-[$1^{-13}$C]Phe-glyoxal | 205.2  | 4.50   | 9.99   | 25 |
| Z-Ala-Pro-[$2^{-13}$C]Phe-glyoxal | 104.3  |       | 9.74   | 4  |
| Z-Ala-Pro-[$2^{-13}$C]Phe-glyoxal | 104.3  |       | 9.19   | 25 |
| Z-Ala-Ala-[$2^{-13}$C]Phe-glyoxal | 104.3  |       | 8.92   | 25 |

**TABLE 4**
Titration constants obtained from pH dependent changes in the chemical shifts of the hydrogen bonded protons in $\alpha$-chymotrypsin and in $\alpha$-chymotrypsin-glyoxal inhibitor complexes.

| Enzyme       | Inhibitor                        | $\delta_1$ | $\delta_2$ | $\delta_3$ | $\Delta_2-\delta_1$ | $pK_1$ | $\Delta_{3-2}$ | $pK_2$ | °C |
|--------------|----------------------------------|------------|------------|------------|---------------------|-------|---------------|-------|----|
| $\alpha$-Chymotrypsin |                                   |            |            |            |                     |       |               |       |    |
| $\alpha$-Chymotrypsin | Z-Ala-Pro-[$2^{-13}$C]Phe-glyoxal | 18.72      | 18.32      | 17.40      | -0.40              | 4.17  | -0.92         | 8.18  | 25 |
| $\alpha$-Chymotrypsin | Z-Ala-Ala-[$2^{-13}$C]Phe-glyoxal | 18.75      | 18.36      | 17.36      | -0.39              | 4.17  | -1.00         | 8.77  | 25 |
FIGURE 4

(a) 25°C

(b) 4°C

% Maximum intensity of the signal at 107 p.p.m.

pH

% Maximum intensity of the signal at 107 p.p.m.

pH
FIGURE 6

A  Z-Ala-Pro-Phe-glyoxal & α-chymotrypsin at 4 °C

B  Z-Ala-Pro-Phe-glyoxal & α-chymotrypsin at 25 °C

C  Z-Ala-Ala-Phe-glyoxal & α-chymotrypsin at 25 °C
super$^{13}$C- and super$^{1}$H-NMR studies of ionisations and hydrogen bonding in chymotrypsin-glyoxal inhibitor complexes
Edward Spink, Sonya Cosgrove, Louis Rogers, Chandralal Hewage and J. Paul G. Malthouse

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