Oxyanion and Tetrahedral Intermediate Stabilization by subtilisin: detection of a new tetrahedral adduct

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ABSTRACT

The peptide-derived glyoxal inhibitor Z-Ala-Ala-Phe-glyoxal has been shown to be ~10 fold more effective as an inhibitor of subtilisin than Z-Ala-Pro-Phe-glyoxal. Signals at 107.2 p.p.m. and 200.5 p.p.m. are observed for the glyoxal keto and aldehyde carbons of the inhibitor bound to subtilisin, showing that the glyoxal keto and aldehyde carbons are sp^3 and sp^2 hybridized respectively. The signal at 107.2 p.p.m. from the carbon atom attached to the hemiketal oxyanion is formed in a slow exchange process that involves the dehydration of the glyoxal aldehyde carbon. Two additional signals are observed one at 108.2 p.p.m. and the other at 90.9 p.p.m. for the glyoxal keto and aldehyde carbons respectively at pHs 6-8 demonstrating that subtilisin forms an additional tetrahedral adduct with Z-Ala-Ala-Phe-glyoxal in which both the glyoxal keto and aldehyde carbons are sp^3 hybridised. For the first time we can quantify oxyanion stabilisation in subtilisin. We conclude that oxyanion stabilisation is more effective in subtilisin than in chymotrypsin. Using ^{1}H-NMR we show that the binding of Z-Ala-Ala-Phe-glyoxal to subtilisin raises the pK_a of the imidazolium ion of the active site histidine residue promoting oxyanion stabilisation. The mechanistic significance of these results are discussed.

1. Introduction

Catalysis by the serine proteases is thought to proceed via a tetrahedral intermediate formed by the addition of the hydroxy oxygen of the active site serine to the carbonyl carbon of the substrate peptide bond undergoing hydrolysis. X-ray crystallographic studies had led to the suggestion that the oxyanion of the tetrahedral intermediate might be stabilised by hydrogen bonding to the backbone amide groups of serine-195 and glycine-193 [1, 2]. Subsequently there has been considerable interest in determining how the oxyanion is stabilised [1-11]. Using ^{13}C-NMR it has been shown that when substrate derived chloromethylketone inhibitors alkylate the active site histidine residue of the serine proteases trypsin [12, 13], chymotrypsin [10, 11, 14-16] and subtilisin [10, 11, 16] the active site serine hydroxy group reacts with ketone carbon to form a tetrahedral adduct analogous to the catalytic tetrahedral intermediate. Oxyanion pK_a values were 2 pK_a units lower in subtilisin chlomethylketone adducts compared to chymotrypsin chloromethylketone inhibitor adducts suggesting that oxyanion stabilisation is more effective in subtilisin compared to chymotrypsin [16]. However, X-ray crystallographic studies have shown that the alkylation of the active site histidine by the chloromethylketone inhibitor causes significant changes in the structure of the tetrahedral adduct compared to tetrahedral adducts which do not alkylate the active site histidine [17, 18]. Recent studies using Z-Ala-Pro-Phe-glyoxal with chymotrypsin have shown that the active site serine hydroxy group reacts with glyoxal keto-carbon to form a tetrahedral hemiketal [19] which is expected to have a structure analogous to the tetrahedral intermediate formed during substrate catalysis. This therefore can be used to estimate oxyanion stabilisation in chymotrypsin. Hemiketal oxyanion formation is a slow exchange process in chymotrypsin involving the conversion of the signal at 100.7 p.p.m. to one at 107.6 p.p.m. and it depends on a pK_a of ~4.5 [20, 21]. With subtilisin and Z-Ala-Pro-Phe-glyoxal a signal at ~107 p.p.m. has also observed but no signals due to the conjugate acid at ~100 p.p.m. have been detected [22].

Therefore the oxyanion pK_a could not be measured in the subtilisin-Z-Ala-Pro-Phe-glyoxal inhibitor complex [22]. We have used Z-Ala-Ala-Phe-glyoxal to determine if replacing the proline residue of Z-Ala-Pro-Phe-glyoxal with an alanine residue significantly affects inhibitor binding and also whether it affects the species formed in the enzyme-inhibitor complex.

In the present work we show that two types of tetrahedral adducts are formed when Z-Ala-Ala-Phe-glyoxal is mixed with subtilisin. We determine the structures of these tetrahedral adducts and in one of these complexes we are able for the first time to quantify oxyanion stabilisation in subtilisin. This allows us to compare hemiketal oxyanion stabilisation in subtilisin- and chymotrypsin-glyoxal inhibitor complexes. We also show that the pK_a of the active site histidine residue in subtilisin is raised when Z-Ala-Ala-Phe-glyoxal binds to subtilisin. The mechanistic significance of these results are discussed.

2. Materials and methods

2.1. Materials

L-[1-^{13}C]phenylalanine (99 atom %) was obtained from Cambridge Isotope Laboratories, Inc. (50, Frontage Road, Andover, MA 01810-5413 USA). All other materials were obtained from Sigma-Aldrich Chemical Co., Gillingham, Dorset, U.K.

2.2. Synthesis of Z-Ala-Ala-Phe-glyoxal, Z-Ala-Ala-[2-^{13}C]Phe-glyoxal and Z-Ala-Ala-[1-^{13}C]Phe-glyoxal

These were synthesized as described by Cosgrove et. al., [23].

2.3. Enzyme solutions

Subtilisin BPN' (crystallized and lyophilized) was obtained from Sigma Chemical Co. and the amount of fully active protein (69%) was determined as described by O'Connell et al., [11].

2.4. Inhibition of subtilisin by Z-Ala-Ala-Phe-glyoxal

The inhibition of the subtilisin Carlsberg catalysed hydrolysis of Succinyl-Ala-Ala-Pro-Phe-pna by Z-Ala-Ala-Phe-glyoxal was studied at 25ºC in 0.1 M buffers (potassium formate pH 3.0-4.5, sodium acetate pH 3.8-5.6, potassium phosphate pH 6.2-8.2, sodium borate pH 8.0-10.5) containing 3.3% (v/v) dimethyl sulphoxide. K_i values were estimated when $[S_0] << K_M$. Therefore, the equation for competitive inhibition $d[P]/dt=k_{cat}*[E]*[S]/([S]+ K_M*(1+[I]/ K_i))$ reduces to $d[P]/dt=(k_{cat}/K_M) *[E]*[S]* K_i/([I]+ K_i)$. Ki values were estimated by using a nonlinear least squares regression program[13].

Stock solutions of 75 mM substrate and 253 mM inhibitor were dissolved in dimethyl sulphoxide. Final concentrations of the enzyme, substrate and inhibitor were in the ranges 0.0075-10 μM, 50-100 μM and 0.003-916 μM respectively.

2.5. 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 for ^{13}C-NMR spectroscopy. The ^{13}C-NMR spectral conditions for the samples of subtilisin inhibited by Z-Ala-Ala-[2-^{13}C]Phe-glyoxal at 11.75 T were: 32768 time domain data points; spectral width 240 p.p.m; acquisition time 0.541 s; 6.0 s relaxation delay time; 90

°C pulse angle; 256 transients recorded per spectrum. Waltz- 16 composite pulse ^{1}H decoupling with a BLARH100 amplifier was used with 16 dB attenuation during the relaxation delay to minimise dielectric heating but maintain the Nuclear Overhauser Effect. Samples of subtilisin inhibited by Z-Ala-Ala-[1-^{13}C]Phe-glyoxal were examined under the same conditions except that the acquisition time was 0.135 s, the relaxation delay time was 0.65 s and 2320 transients were recorded per spectrum. Unless stated otherwise all spectra were transformed using an exponential weight factor of 20 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 subtilisin inhibited by Z-Ala-Ala-Phe-glyoxal at 11.75 T were: 32768 time-domain data points; spectral width 40 p.p.m.; acquisition time 0.41 s; 0.4 s relaxation delay time; 90° pulse angle; 512 transients were recorded per spectrum. Water suppression was achieved using the Watergate W5 pulse sequence with gradients [24]. Spectra were transformed using an exponential weighting factor of 50 Hz. ^{13}C-NMR spectra confirming the formation of the subtilisin 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 [14]. The chemical shift d_6-dimethyl sulphoxide (< 5% (v/v)) at 38.7 in p.p.m. was also used as a secondary reference in aqueous solutions. All aqueous samples contained 10% (v/v) $^{2}H_2O$ to obtain a deuterium lock signal, as well as 10 mM potassium phosphate buffer to help maintain stable pH values during the pH titration's.

2.6. Calculation of pK_a values

The pK_avalues of hemiketal hydroxyl groups were calculated using the relationship pK_a= 15.9 – 1.42 S σ* [25]. The σ* values used were 0.57 for the alpha-carbon [26] attached to the

glyoxal hemiketal carbon. For the serine hydroxy group (OR) of the hemiketal a value of $\sigma^* = 1.68$ was calculated ($6.23 \times \sigma_I$) from the σ_I value of 0.27 [27]. σ^* values of 2.15 and 1.37 were used for the glyoxal aldehyde (-CHO) and its hydrate ($-CH(OH)_2$) groups respectively [28].

3. Results

3.1. Inhibition of subtilisin Carlsberg by Z-Ala-Ala-Phe-glyoxal

K_i values were estimated at 25˚C when $[S_0] << K_M$ and $d[P]/dt = (k_{cat}/K_M)*[E]*[S_0]*K_i/([I]+K_i)$. For Z-Ala-Ala-Phe-glyoxal binding to subtilisin Carlsberg a K_i value of 0.069 ± 0.003 μM was obtained at pH 7.2 (Table 1). However, the binding of Z-Ala-Pro-Phe-glyoxal by both subtilisin BPN' and subtilisin Carlsberg was much weaker. For subtilisin BPN' a K_i value of 5.32 ± 0.34 μM at pH 7.02 has been reported [22] and in the present work a similar K_i value of 0.96 ± 0.06 μM was obtained at pH 7.06 with subtilisin Carlsberg. Therefore we conclude that replacing proline with alanine significantly improves inhibitor binding. The K_i values for the inhibition of subtilisin Carlsberg by Z-Ala-Ala-Phe-glyoxal increased at higher and lower pHs (Table 1). Similar increases in K_i values were observed when chymotrypsin was inhibited by either Z-Ala-Pro-Phe-glyoxal [20, 21] or Z-Ala-Ala-Phe-glyoxal [21].

3.2. ^{13}C NMR of subtilisin inhibited by Z-Ala-Ala-[1-^{13}C]Phe-glyoxal or by Z-Ala-Ala-[2-^{13}C]Phe-glyoxal

Z-Ala-Ala-[2-^{13}C]Phe-glyoxal in water gave signals at 206.7 p.p.m. and 96.5 p.p.m. (Fig. 1a) due to the glyoxal keto group (Scheme 1, structure (b)) and its hydrate (Scheme 1, structure (a)) respectively. The signal at 38.7 p.p.m. (Fig 1a,e) is due to d_6-dimethyl

sulphoxide which was used as a solvent for the glyoxal inhibitor. In the presence of excess subtilisin at pH 7.2 (Fig. 1b) these signals were replaced by signals at 108.2 p.p.m. and 107.2 p.p.m. (Fig. 1c). On adding an excess of Z-Ala-Ala-[2-^{13}C]Phe-glyoxal these signals reached a maximum intensity and additional signals due to the excess free inhibitor were observed at 206.7 p.p.m. and 96.5 p.p.m. (Fig. 1d). The new signals at 107.2 p.p.m. and 108.2 p.p.m. observed at pH 7.2 (Fig. 1c, d) are assigned to enzyme bound species.

In aqueous solution Z-Ala-Ala-[1-^{13}C]Phe-glyoxal had signals at 88.8 p.p.m. and 90.2 p.p.m. (Fig. 1e) due to the hydrated glyoxal aldehyde group in the presence of the glyoxal keto group (Scheme 1, structure (b)) and its hydrate (Scheme 1, structure (a)) respectively in water. On adding Z-Ala-Ala-[1-^{13}C]Phe-glyoxal to subtilisin at pH 6.9 new signals at 90.9 p.p.m. and 200.5 p.p.m. were observed (Fig. 1f). On adding an excess of Z-Ala-Ala-[1-^{13}C]Phe-glyoxal these signals reached a maximum intensity and additional signals due to the excess free inhibitor were observed at 90.2 p.p.m. and 88.8 p.p.m. (Fig. 1g). The new signals at 90.9 p.p.m. and 200.5 p.p.m. are assigned to an enzyme bound species.

The intensity of the signals due to the enzyme bound species (Structures (c)-(f) in Scheme 1) at 90.9 p.p.m. (Fig. 2B), 107.2 p.p.m. (Fig. 3B), 105.0-108.2 p.p.m. (Fig. 3B), and 200.5 p.p.m. (Fig. 2A) all decreased at low pH as the K_i values increased (Table 1) and there was a concomitant increase in the intensity of the signals at 88.8 p.p.m. (Fig. 2B), 90.2 (Fig. 2B), 96.5 p.p.m. (Fig. 3C) and 206.7 p.p.m. (Fig. 3A) due to free inhibitor (Structures (a) and (b) in Scheme 1).

Similar signals to those at 107.2 p.p.m. and 200.5 p.p.m. have been observed when Z-Ala-Pro-[2-^{13}C]Phe-glyoxal and Z-Ala-Pro-[1-^{13}C]Phe-glyoxal respectively bind to subtilisin [22] or chymotrypsin [20, 21]. The signal at ~107 pm was assigned to the oxyanion form of the hemiketal carbon [19] and the signal at ~200 p.p.m. was assigned to the non-hydrated aldehyde carbon (Structure (e) in Scheme 1). We assign the signals at 107.2 p.p.m. and 200.5 p.p.m. observed in the present study to the same structure in the subtilisin-Z-Ala-Ala-Phe-

glyoxal complex (Structure (e) in Scheme 1). The additional signals observed at 108.2 p.p.m. and 90.9 p.p.m. using Z-Ala-Ala-[2-^{13}C]Phe-glyoxal and Z-Ala-Ala-[1-^{13}C]Phe-glyoxal respectively show that an additional species is formed also with an ionised hemiketal hydroxyl group (108.2 p.p.m.) and a hydrated aldehyde group (90.9 p.p.m.) (Structure (f) in Scheme 1).

The chemical shift of the signal at 107.2 p.p.m. did not change with pH (Fig. 3B). However its intensity decreased at high or low pH values (Fig. 3B) as has been observed with chymotrypsin-glyoxal inhibitor complexes [21]. But, with chymotrypsin-glyoxal inhibitor complexes the decrease in intensity on decreasing the pH led to the concomitant increase in the intensity of a signal that titrated from ~100 to ~104 p.p.m. [20, 21]. However, as in our earlier study with the Z-Ala-Pro-Phe-glyoxal and subtilisin the signals at 100 to 104 p.p.m. were not observed with Z-Ala-Ala-[2-^{13}C]Phe-glyoxal and subtilisin (Fig. 3B) instead there was a concomitant increase in the intensity of the signals at 206.7 p.p.m. (Fig. 3A) and 96.5 p.p.m. (Fig. 3C) as the pH decreased due to the free inhibitor.

The signal at 108.2 p.p.m. (Fig. 3B) is formed by the ionization of the hemiketal (Structure (c) in Scheme 1). Therefore there is no slow dehydration process when the hemiketal oxyanion of the signal 108.2 p.p.m. is formed (Structure (f) in Scheme 1) and as this is a fast exchange process the chemical shift of this signal is pH dependent (Fig. 4A) titrating from 100.80 p.p.m. to 108.27 p.p.m. with a pK_a of 3.93 ± 0.02. This titration shift of 7.53 p.p.m. is similar to that (6.87 p.p.m. [20] and 7.01 p.p.m. [21]) observed for the hemiketal ionisation by slow exchange in the Z-Ala-Pro-Phe-glyoxal-chymotrypsin complex. As we have obtained data from pH 3.56 to 8 which encompasses the pK_a of 3.9 (Fig. 4A) we believe that we have obtained a good estimate of the titration constants for the fast exchange process using Z-Ala-Ala-[2-^{13}C]Phe-glyoxal. The signal at 90.9 p.p.m. in the subtilisin-Z-Ala-Ala-[1-^{13}C]Phe-glyoxal complex titrated with a similar pK_a of 4.09 but with a much smaller titration shift (Fig. 4B) as expected if it reflects the titration of the hemiketal oxyanion

(Structures (c) and (f) in Scheme 1). Subtilisin undergoes denaturation due to autolysis at alkaline pH values where it is catalytically active. However, at low pH values it has a low catalytic activity reducing the rate of autolysis.. Also inhibitor binding should protect against autolysis and could help maintain the enzyme in an active conformation [22]. Therefore extensive denaturation is not expected in our NMR experiments at acid pH values. To confirm this the intensity of the signals at 108.2 and 107.2 at pH 7.0 (spectrum (c) in Fig. 3B) were compared before and after incubating at pH 3.59 for 40 minutes. There was only an ~ 20% decrease in signal intensity and no new signals due to denatured species were observed. Therefore we conclude that the pK_a of ~ 4 is not due to irreversible denaturation.

3.3. ^{1}H-NMR of the the hydrogen bonded protons of the Z-Ala-Ala-Phe-glyoxal-Subtilisin inhibitor complex.

At pH values from 3.77 to pH 6.50 two signals were observed one at 13.1 p.p.m. and the other at 18.9 p.p.m. (Fig. 6 (a-e)). Similar signals at ~18.9 p.p.m. and ~13.1 p.p.m. have been observed in chymotrypsin-glyoxal inhibitor complexes [21] and in chymotrypsin-trifluoromethyl ketone inhibitor complexes [29]. The signals at 13.1 p.p.m. and 18.9 p.p.m. are assigned in the same way to the N^{1}_{δ} and N^{2}_{ϵ} protons respectively of the imidazolium ion of the active site histidine in the Z-Ala-Ala-Phe-glyoxal-subtilisin complex (Scheme 2). The absence of a signal at ~ 15 p.p.m. due to the N^{1}_{δ} proton of imidazole [30] and the presence of the signals at ~13 and ~19 p.p.m. due to the N^{1}_{δ} and N^{2}_{ϵ} protons of the imidazolium ion of histidine-64 shows that when the glyoxal inhibitor is bound to subtilisin the active site histidine residue (Histidine-64) is fully protonated and its pK_a is greater than 10.5 (Fig. 6).

The intensity of these signals decreased at low pHs (Fig. 6) corresponding to the decrease in the K_i values of the Z-Ala-Ala-Phe-glyoxal-subtilisin inhibitor complex at low pH (Table 1)). A decrease in signal intensity was also observed at alkaline pH values Fig. (6(f-i)).

Unfolded subtilisin is rapidly autolysed by catalytically active subtilisin at alkaline pH values [22]. When the sample at pH 10.52 (Fig. 6(i)) was adjusted to pH 6.48 the intensity of the signals at 13.1 and 18.9 p.p.m. were reduced by ~ 60% confirming that significant irreversible denaturation had occurred. The new signals at 18.6 and 13.3 p.p.m. present at pH 10.52 (Fig. 6(i)) were also present at when the pH was lowered to pH 6.48 (spectrum not shown) and are assigned to irreversibly denatured species formed by alkaline denaturation.

4. Discussion

The active site of subtilisin can be described as a shallow groove open on one side to solvent while that of chymotrypsin consists of a more deeply invaginated hydrophobic pocket [16, 31, 32]. This could explain why the glyoxal aldehyde carbon is dehydrated in chymotrypsin inhibitor complexes but it is partially hydrated in the more solvent exposed subtilisin-glyoxal inhibitor complexes. For Z-Ala-Pro-Phe-glyoxal and Z-Ala-Ala-Phe-glyoxal the glyoxal aldehyde groups have K_{HYD} values of ~90 in water [19]. Therefore the glyoxal aldehyde group is hydrated in aqueous solutions. However, when Z-Ala-Ala-Phe—glyoxal is bound to subtilisin K_{HYD} is ~1 and when Z-Ala-Pro-Phe-glyoxal is bound to subtilisin K_{HYD} is << 1. Therefore binding energy is being used to dehydrate the hydrated glyoxal aldehyde group. Therefore if binding did not result in dehydration of the glyoxal aldehyde carbon there could be at least a 100 fold increase in inhibitor potency. The fact that Z-Ala-Ala-Phe-glyoxal is bound 14 fold tighter to subtilisin Carlsberg than to Z-Ala-Pro-Phe-glyoxal could in part at least be explained by the fact that on binding to subtilisin the glyoxal aldehyde carbon of Z-Ala-Ala-Phe-glyoxal is less dehydrated than the glyoxal aldehyde carbon of Z-Ala-Pro-glyoxal. The most effective glyoxal inhibitor we have studied with a serine protease was Z-Ala-Pro-Phe-glyoxal which had a Ki of 33 nM with chymotrypsin [21]. However, in this case the aldehyde carbon of the glyoxal inhibitor was fully dehydrated when it was bound to chymotrypsin. Therefore this suggests that with serine proteases which do not dehydrate the glyoxal aldehyde carbon even more potent inhibition could be obtained. It is clear that the full potency of glyoxal inhibitors has not yet been utilised with the serine proteases and so glyoxal inhibitors may be even more effective with other serine proteases which do not dehydrate the glyoxal aldehyde carbon.

In the subtilisin-BPN'-Z-Ala-Pro-[2-^{13}C]Phe-glyoxal complex the signal at 107.2 p.p.m. due to the hemiketal oxyanion carbon (Structure (e) in Scheme 1) was observed but the

signal at ~100 to 104 p.p.m. due to its conjugate acid (Structure (c) and (d) in Scheme 1) was not observed [22]. Likewise in this study with subtilisin Carlsberg inhibited by Z-Ala-Ala-[2-^{13}C]Phe-glyoxal the signal at 107.2 p.p.m. is observed but no signal at 100 - 104 p.p.m. was observed. It has been shown that in chymotrypsin- or subtilisin-chloromethylketone inhibitor tetrahedral adducts the oxyanion pK_a values are ~2 pK_a units smaller in the subtilisin-chloromethylketone adducts compared to chymotrypsin adducts [11, 16, 33]. Therefore it has been argued [22] that the hemiketal oxyanion in species (e) in Scheme 1 in the subtilisin-BPN'-Z-Ala-Pro-Phe-glyoxal complex will have a pK_a of ~ 2.5 at least 2 pK_a units lower than that observed (pK_a ~ 4.5) in chymotrypsin -glyoxal inhibitor complexes [20, 21]. This would explain why a signal at 100-104 p.p.m. due to the carbon of the non-ionised hemiketal structures (c) and (g) in Scheme 1 has not been observed with subtilisin in both the present study using Z-Ala-Ala-Phe-glyoxal and in the earlier study using Z-Ala-Pro-Phe-glyoxal [22]. Such a species is observed in chymotrypsin-glyoxal complexes and it is formed as the signal at ~107 p.p.m. is lost. Therefore these signals are in slow exchange. In the subtilisin-Z-Ala-Ala-[2-^{13}C]Phe-glyoxal complex the chemical shift of the signal at ~107 did not change with pH but its intensity decreased at acid pHs according to a pK_a of ~4 (Fig. 5A) and it was not observed at low pH (Fig. 3B). A similar result was obtained with the subtilisin-Z-Ala-Pro-Phe-glyoxal complex [22]. In both cases the intensity of the signal decreased as the pH decreased. The intensity signal at ~107 p.p.m. also decreased at alkaline pHs (Fig. 3B) according to a pK_a of ~7.8 (Fig. 5A). The intensity of the signal at 200.5 p.p.m. in the subtilisin-Z-Ala-Ala-[1-^{13}C]Phe-glyoxal complex also had a bell shaped pH dependence on pK_a values of ~4 and ~8 (Fig. 5B) confirming that both signals were in the same species (Structure (e) in Scheme 1). The hydration of aldehyde carbonyl groups is a slow reaction subject to general acid-base catalysis [34] and so the bell shaped pH dependence for the concentration of structure (e) in Scheme 1 is expected due to the hydration of its aldehyde group at high and low pH [21].

From following the interconversion of the two structures (c) and (e) (Scheme 1) by slow exchange a pK_a of 4.5 has been attributed to the hemiketal oxyanion in chymotrypsin [20, 21]. However, this is a three step process involving the species (c), (d) and (e) (Scheme 1). For the interconversion of structures (c) and (e) in Scheme 1, $K_{obs} = [e].[H^+]/[c]$, $K_a = [e].[H^+]/[d]$ and $K_{HYD} = [c]/[d]$. Therefore $K_{obs} = K_a/K_{HYD}$ and $pK_{obs} = pK_a + \log_{10} K_{HYD}$. pK_{obs} was 4.5 for the hemiketal oxyanion in Z-Ala-Pro-Phe-glyoxal complexes with δ-chymotrypsin [20] and α-chymotrypsin [21]. For Z-Ala-Pro-Phe-glx in water a value of 89.3 has been determined for the K_{HYD} of the glyoxal aldehyde group in water [19]. If this value is the same for the glyoxal aldehyde group in the hemiketal species (Structures (c) and (d) in Scheme 1) then the oxyanion pK_a will be significantly different from pK_{obs} ($pK_a = pK_{obs}$-1.95) in structures (d) and (e) in Scheme 1. However, in chymotrypsin only the dehydrated form (Structure (e) in Scheme 1) of the ionised hemiketal was detected [20, 21]. Therefore in this case no correction to pK_{obs} is required and so the hemiketal oxyanion $pK_a = pK_{obs} = 4.5$ for structures (d) and (e) in Scheme 1. With subtilisin a pK_a value of 4.0 was determined (Table 2) from the interconversion of the two structures (c) and (f) in Scheme 1 by fast exchange (Fig. 4). In subtilisin the signals from both the hydrated (108.2 p.p.m., Fig. 3B) and dehydrated (107.2 p.p.m., Fig. 3B)) forms of ionised hemiketals (Structures (e) and (f) in Scheme 1) have similar intensities (Fig. 3B) and so their K_{HYD} values will be ~1 and therefore pK_{obs} (for structure (e) in Scheme 1) = $pK_a + \log_{10} K_{HYD} = pK_a$ (Table 2).

Using the free energy relationship $pK_a = 15.9 - 1.42\ \Sigma\sigma*$ [25] we estimate that the pK_a value of the hemiketal oxyanion will be 9.64 and 10.74 in structures (e) and (f) respectively of Scheme 1. The pK_a of 10.74 is in good agreement with the value of 10.9 estimated previously for the pK_a of the hemiketal hydroxyl group in structure (c) in Scheme 1 [20] using the free energy relationship of De Tar [35]. In subtilisin the hemiketal ionisation (structures (c) and (f) in Scheme 1) does not involve the slow hydration or dehydration reactions and so it is a fast exchange process that can be used to determine the pK_a of the

hemiketal oxyanion when the glyoxal aldehyde group is hydrated (Structure (f) in Scheme 1). Therefore the pK_a of 4.0 for this hemiketal oxyanion shows that subtilisin lowers the oxyanion pK_a by 6.74 pK_a units (ΔpK_a = 10.74-4.0, Table 2) when the glyoxal aldehyde group is hydrated. Also with subtilisin, a signal due to the conjugate acid at 100-104 p.p.m. (Structures (d) and (g) in Scheme 1) of the hemiketal oxyanion at 107.2 p.p.m. (Structure (e) in Scheme 1) was not observed (Fig. 3B) showing that the oxyanion $pK_a < 3.0$ when the glyoxal aldehyde group is dehydrated [22]. Therefore for subtilisin, hemiketal oxyanion stabilisation (Table 2) is at least as effective the when the glyoxal aldehyde group is dehydrated (structure (e) in Scheme 1) than when it is hydrated (structure (f) in Scheme 1). In chymotrypsin the hemiketal oxyanion pK_a of 4.5 was determined when the glyoxal aldehyde group was dehydrated (structure (e) in Scheme 1) and so oxyanion stabilisation (ΔpK_a = 9.64-4.5 = 5.14, Table 2) is at least 30 fold more effective in subtilisin than in chymotrypsin (Table 2).

In a recent ^{1}H-NMR study of the hydrogen bonded protons of δ-chymotrypsin (Scheme 2) at 5 ˚C the signal at ~18 at low pH has been shown to be composed of two signals one at 18.2 p.p.m. and the other at 17.5 p.p.m. [36]. These were in slow exchange and were assigned to the $N^{\delta 1}$ proton of the imidazolium ion of the histine-57 residue in the presence of the ionised carboxylate of aspartate-102 (18.2 p.p.m.) and of its conjugate acid (17.5p.p.m.). In our study of subtilisin in the presence of Z-Ala-Ala-Phe-glyoxal signals at 18.9 and 18.6 p.p.m. were detected (Fig. 6h). The signal at 18.6 resulted from irreversible denaturation. Therefore the failure to observe a signal at 17.5 p.p.m. confirms that the aspartate residue of the catalytic triad is fully ionised in the subtilisin-glyoxal inhibitor complex (Fig. 6). The signal at 17.5 p.p.m. has not been detected at acid pHs in chymotrypsin-glyoxal inhibitor complexes but it has been observed alkaline pHs 9-11 [21]. As it is unlikely that the aspartate group would become protonated at high pH values we believe it is unlikely that the signal at 17.5 p.p.m. in the chymotrypsin-glyoxal inhibitor adducts results from formation of the conjugate acid of the aspartate-102 residue.

It is however clear that as with chymotrypsin [21] binding of glyoxal inhibitors to subtilisin raises the pK_a of histidine-57 to a value >10.5. It has been suggested that the binding of substrates or structurally related inhibitors will induce a conformational change resulting in steric compression between histidine-57 and aspartate-102 and the formation of a low barrier hydrogen bond raising the pK_a of histidine-57 [37, 38]. Recent calculations [39, 40] support the proposal [15, 41] that inhibitor or substrate binding causes desolvation of the active site histidine residue in the serine proteases raising the pK_a of the histidine residue and allowing it to be an effective general base catalyst enhancing the nucleophilicity of the hydroxyl group of serine-195 [15, 21, 33, 41]. Raising the histidine pKa also allows it to act as a general acid catalyst for the breakdown of the tetrahedral intermediate [13-15, 21, 42] and to stabilize oxyanion formation [15, 21]. Our results show that the binding of glyoxal inhibitors also raises the pKa of the active site histidine in subtilisin promoting oxyanion stabilization. Therefore in both subtilisin and chymotrypsin substrate binding is expected to raise the histidine pK_a allowing it to be an effective general base catalyst enhancing the nucleophilicity of the hydroxyl group of the active site serine and also acting as a general acid catalyst for the breakdown of the tetrahedral intermediate during catalysis.

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SCHEMES

Scheme 1. Chemical shifts and structures of Z-Ala-Ala-Phe-Glyoxal in the presence and absence of subtilisin Carlsberg**.** Structure (g) was only observed in chymotrypsin-glyoxal inhibitor complexes. Structure (d) has not been observed in subtilisin- or chymotrypsin-glyoxal inhibitor complexes.

Scheme 2. Chemical shifts and structures of the hydrogen-bonded protons observed when Z-Ala-Ala-Phe-Glyoxal is incubated in the presence and absence of subtilisin Carlsberg

FIGURE LEGENDS

Fig. 1. ^{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 addition to subtilisin**.** Acquisition and processing parameters were as described in the Materials and methods section except that for spectrum (e) 1024 transients were recorded. Sample conditions were: (a) 3.00 ml of 0.66 mM Z-Ala-Ala-[2-^{13}C]Phe Glyoxal, 3.3% (v/v) d_6-dimethyl sulphoxide, pH=7.03; (b) 2.90 ml of 0.92 mM subtilisin, pH=7.01; (c) 2.92 ml of 0.95 mM subtilisin, 1.2 mM Z-Ala-Ala-[2-^{13}C]Phe Glyoxal, 0.58 % (v/v) d_6-dimethyl sulphoxide, pH=6.96 (d) 2.93 ml of 0.92 mM subtilisin, 2.07 mM Z-Ala-Ala-[2-^{13}C]Phe Glyoxal, 1.0% (v/v) d_6-dimethyl sulphoxide, pH=6.98 (e) 3.00 ml of 0.74 mM Z-Ala-Ala-[1-^{13}C]Phe Glyoxal, 3.3% (v/v) d_6-dimethyl sulphoxide, pH=7.19; (f) 2.92 ml of 0.99 mM subtilisin, 0.76 mM Z-Ala-Ala-[1-^{13}C]Phe Glyoxal, 0.68 % (v/v) d_6-dimethyl sulphoxide, pH=7.25: (g) 2.95 ml of 1.04 mM subtilisin, 1.89 mM Z-Ala-Ala-[1-^{13}C]Phe Glyoxal, 1.69 % (v/v) d_6-dimethyl sulphoxide, pH=7.19. All samples contained 10% (v/v) ^2H_2O and 10mM potassium phosphate buffer.

Fig. 2. Effect of pH on the ^{13}C-NMR signals from Z-Ala-Pro-[1-^{13}C]Phe Glyoxal in the presence of subtilisin. Acquisition and processing parameters were as described in the Materials and methods section. For spectrum (c) the sample contained 2.95 ml of 1.89 mM Z-Ala-Ala-[1-^{13}C]Phe Glyoxal containing 0.97 mM subtilisin at pH = 7.23. The volumes of 1M HCl containing 10% (v/v) $^{2}H_2O$ that were added to the samples for spectra d-j were 0.018, 0.013, 0.005, 0.007, 0.007, 0.010, 0.020 ml, respectively. Spectrum (b) was obtained using a new 2.97 ml sample containing 1.88 mM Z-Ala-Ala-[1-^{13}C]Phe Glyoxal containing 1.01 mM subtilisin at pH = 8.00. The sample for spectrum (a) was obtained by adding 0.03 ml of 1M KOH containing 10% (v/v) $^{2}H_2O$.

Fig. 3. Effect of pH on the ^{13}C-NMR signals from Z-Ala-Pro-[2-^{13}C]Phe Glyoxal in the presence of subtilisin**.** Acquisition and processing parameters were as described in the Materials and methods section. For spectrum (c) the sample contained 2.95 ml of 1.88 mM Z-Ala-Ala-[1-^{13}C]Phe Glyoxal containing 0.92 mM subtilisin and 1.53% (v/v) d_6-dimethyl sulphoxide at pH = 6.96. The volumes of 1M HCl containing 10% (v/v) $^{2}H_2O$ that were added to the samples for spectra d-j were 0.012, 0.012, 0.010, 0.010, 0.012, 0.012, 0.015 ml, respectively. Spectrum (b) was obtained using a new 2.96 ml sample containing 1.87 mM Z-Ala-Ala-[1-^{13}C]Phe Glyoxal containing 0.91 mM subtilisin and 1.53% (v/v) d_6-dimethyl sulphoxide at pH = 8.03. The sample for spectrum (a) was obtained by adding 0.013 ml of 1M KOH containing 10% (v/v) $^{2}H_2O$.

Fig. 4. pH titrations of the chemical shift values of the ^{13}C-NMR signals in the complexes formed between subtilisin and Z-Ala-Ala-[1-^{13}C]Phe Glyoxal and Z-Ala-Ala-[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. (1) and the appropriate fitted parameters given below.

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

The fitted parameters were (a) Subtilisin and Z-Ala-Ala-[2-^{13}C]Phe Glyoxal: $pK_a = 3.93 \pm 0.02$, $S_1 = 100.8 \pm 0.18$ p.p.m. and $S_2 = 108.27 \pm 0.18$ p.p.m.; (b) Subtilisin and Z-Ala-Ala-[1-^{13}C]Phe Glyoxal: $pK_a = 4.09 \pm 0.08$, $S_1 = 89.97 \pm 0.06$ p.p.m. and $S_2 = 90.91 \pm 0.02$ p.p.m.

Fig. 5. Effect of pH on the intensity of the ^{13}C-NMR signals at 107.2 p.p.m. and 200.5 p.p.m. in the complexes formed between subtilisin and Z-Ala-Ala-[2-^{13}C]Phe Glyoxal and Z-Ala-Ala-[1-^{13}C]Phe Glyoxal respectively. Acquisition parameters and sample conditions were as described in the Materials and methods section. The continuous lines were calculated using eqn. (2) and the appropriate fitted parameters given below.

$I_{obs} = I_{max} / (1 + [H]/K_1 + K_2/[H])$ (2)

The fitted parameters were (a) Signal at 107.2 p.p.m. in the subtilisin and Z-Ala-Ala-[2-^{13}C]Phe Glyoxal complex: $pK_1 = 4.31 \pm 0.14$, $pK_2 = 7.89 \pm 0.22$, and $I_{max} = 105.6 \pm 9.2\%$; (b) Signal at 200.5 p.p.m. in the subtilisin and Z-Ala-Ala-[1-^{13}C]Phe Glyoxal complex: $pK_1 = 3.78 \pm 0.13$, $pK_2 = 8.09 \pm 0.16$, and $I_{max} = 100.7 \pm 5.7\%$.

Fig. 6. Effect of pH on the ^{1}H-NMR signals from Z-Ala-Pro-[2-^{13}C]Phe Glyoxal in the presence of subtilisin. Acquisition and processing parameters were as described in the Materials and methods section. For spectrum (e) the sample contained 0.98 ml of 2.02 mM Z-Ala-Ala-[2-^{13}C]Phe Glyoxal containing 1.00 mM subtilisin and 1.64% (v/v) d_6-dimethyl sulphoxide at pH = 6.50. The volumes of 1M HCl containing 10% (v/v) $^{2}H_2O$ that were added to the samples for spectra d-a were 0.007, 0.005, 0.008, 0.015 ml, respectively. Spectrum (f) was obtained using a new 0.986 ml sample containing 2.00 mM Z-Ala-Ala-[2-^{13}C]Phe Glyoxal containing 1.14 mM subtilisin and 1.60 % (v/v) d_6-dimethyl sulphoxide at pH = 7.52. The volumes of 1M KOH containing 10% (v/v) $^{2}H_2O$ that were added to the samples for spectra g-i were 0.005, 0.004, 0.005, ml, respectively.

Table 1

Disassociation constants for the Z-Ala-Ala-Phe-glyoxal-Subtilisin Carlsberg complexes from pH 3.43 to pH 8.90

pH	$K_{d(obs)}$ (μM)	pH	$K_{d(obs)}$ (μM)
3.43	179.0	7.20	0.069
4.57	7.76	8.15	0.550
5.85	0.31	8.90	0.531

Table 2

Oxyanion stabilisation by subtilisin and chymotrypsin

Enzyme	Enzyme inhibitor complex	Oxyanion pK_a		$pK_{ae} - pK_{aw}$	ΔG (kJ.mol^{-1})
		pK_{aw} [a] (water)	pK_{ae} [a] (enzyme)		
Subtilisin	Structure (f) in Scheme 1	10.74	4.0	-6.74	-38.5
Subtilisin	Structure (e) in Scheme 1	9.64	< 3.0	>6.64	>37.9
Chymotrypsin	Structure (e) in Scheme 1	9.64	4.5[b]	-5.14	-29.3

[a] 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.

[b] Data from [20, 21].

Fig1

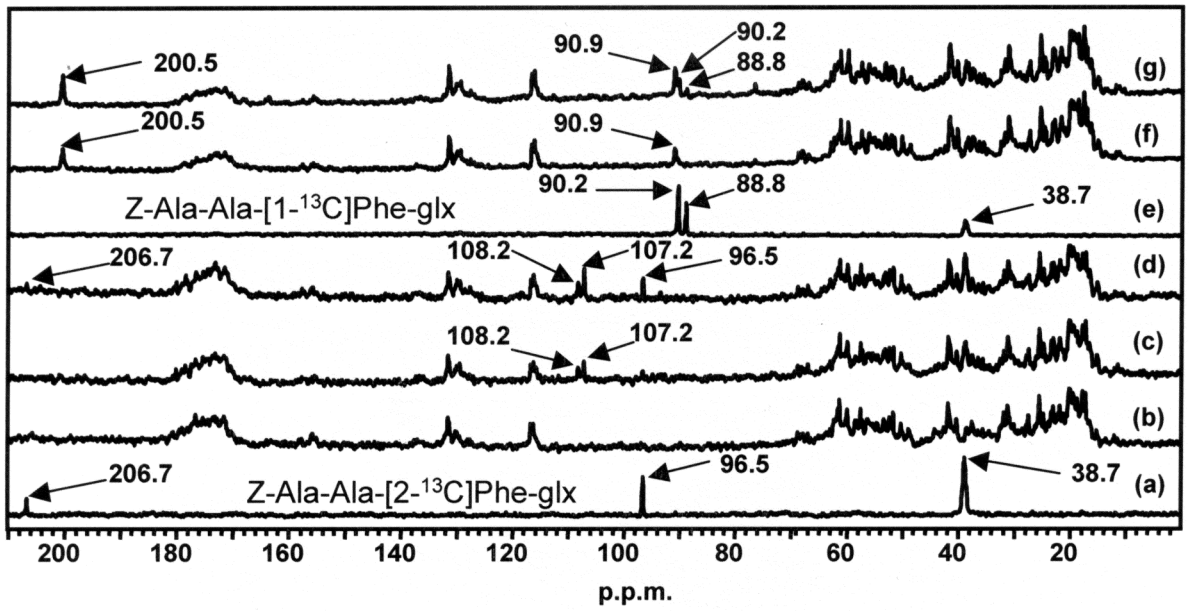

200.5
90.9
90.2
88.8
(g)
200.5
90.9
(f)
Z-Ala-Ala-[1-13C]Phe-glx
90.2
88.8
38.7
(e)
206.7
108.2
107.2
96.5
(d)
108.2
107.2
(c)
(b)
206.7
Z-Ala-Ala-[2-13C]Phe-glx
96.5
38.7
(a)
200
180
160
140
120
100
80
60
40
20
p.p.m.

Fig2

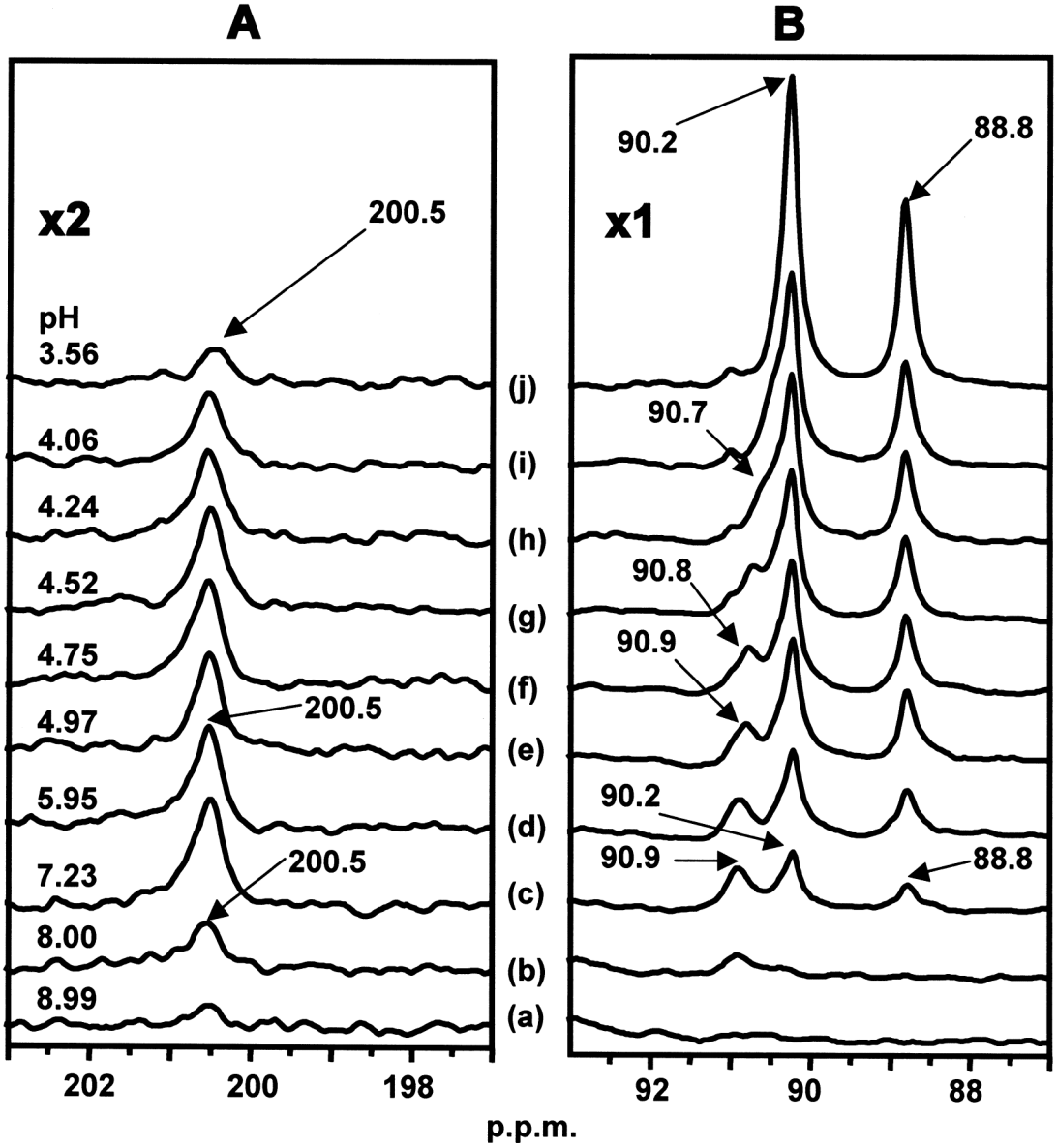

A
B
x2
x1
200.5
pH
3.56
4.06
4.24
4.52
4.75
4.97
5.95
7.23
8.00
8.99
(j)
(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
90.2
88.8
90.7
90.8
90.9
202
200
198
92
90
88
p.p.m.

Fig3

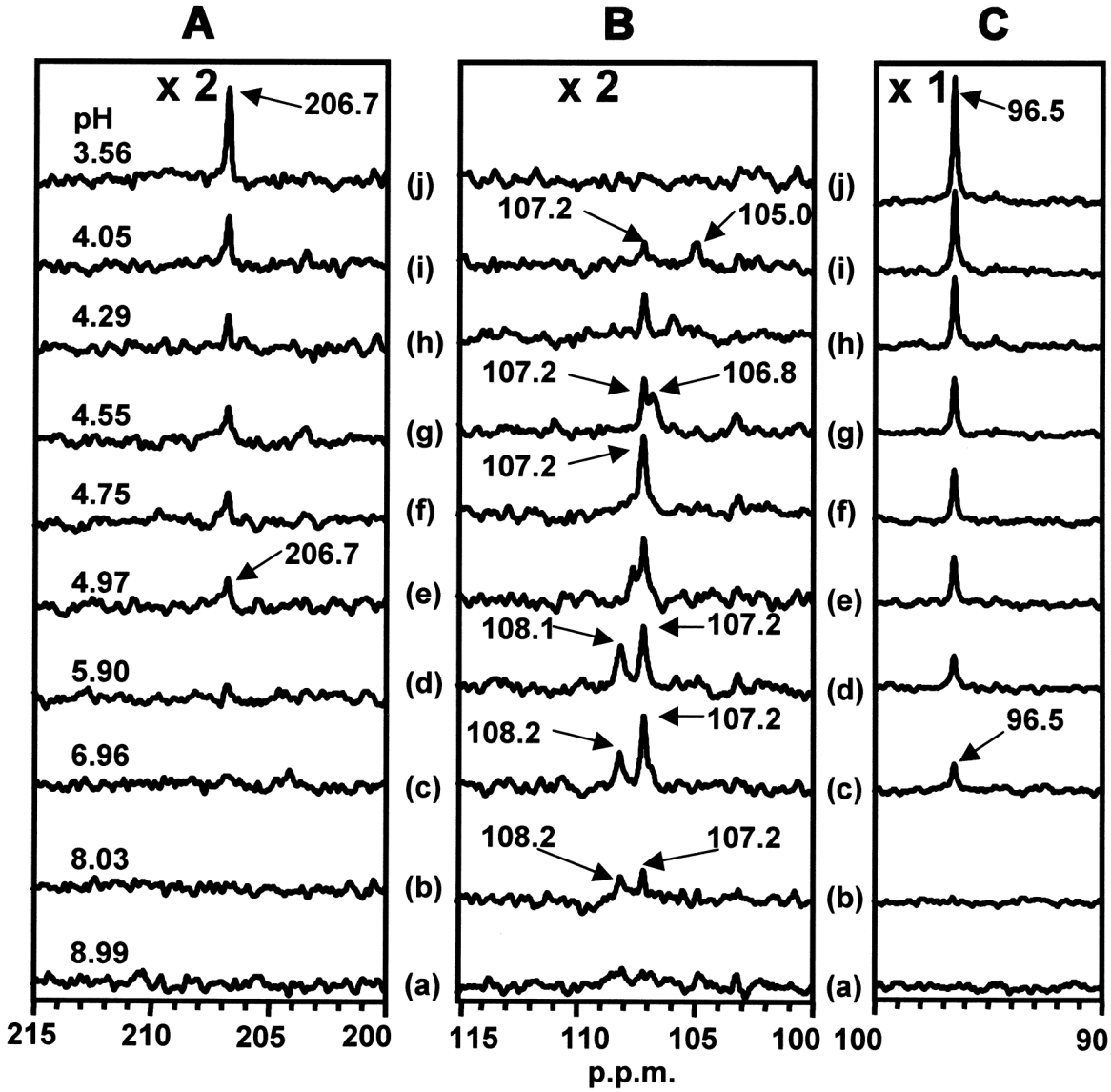

A
B
C
x 2
x 2
x 1
206.7
96.5
pH
3.56
4.05
4.29
4.55
4.75
4.97
5.90
6.96
8.03
8.99
(j)
(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
107.2
105.0
107.2
106.8
107.2
108.1
107.2
108.2
107.2
108.2
107.2
206.7
96.5
215
210
205
200
115
110
105
100
100
90
p.p.m.

Fig4

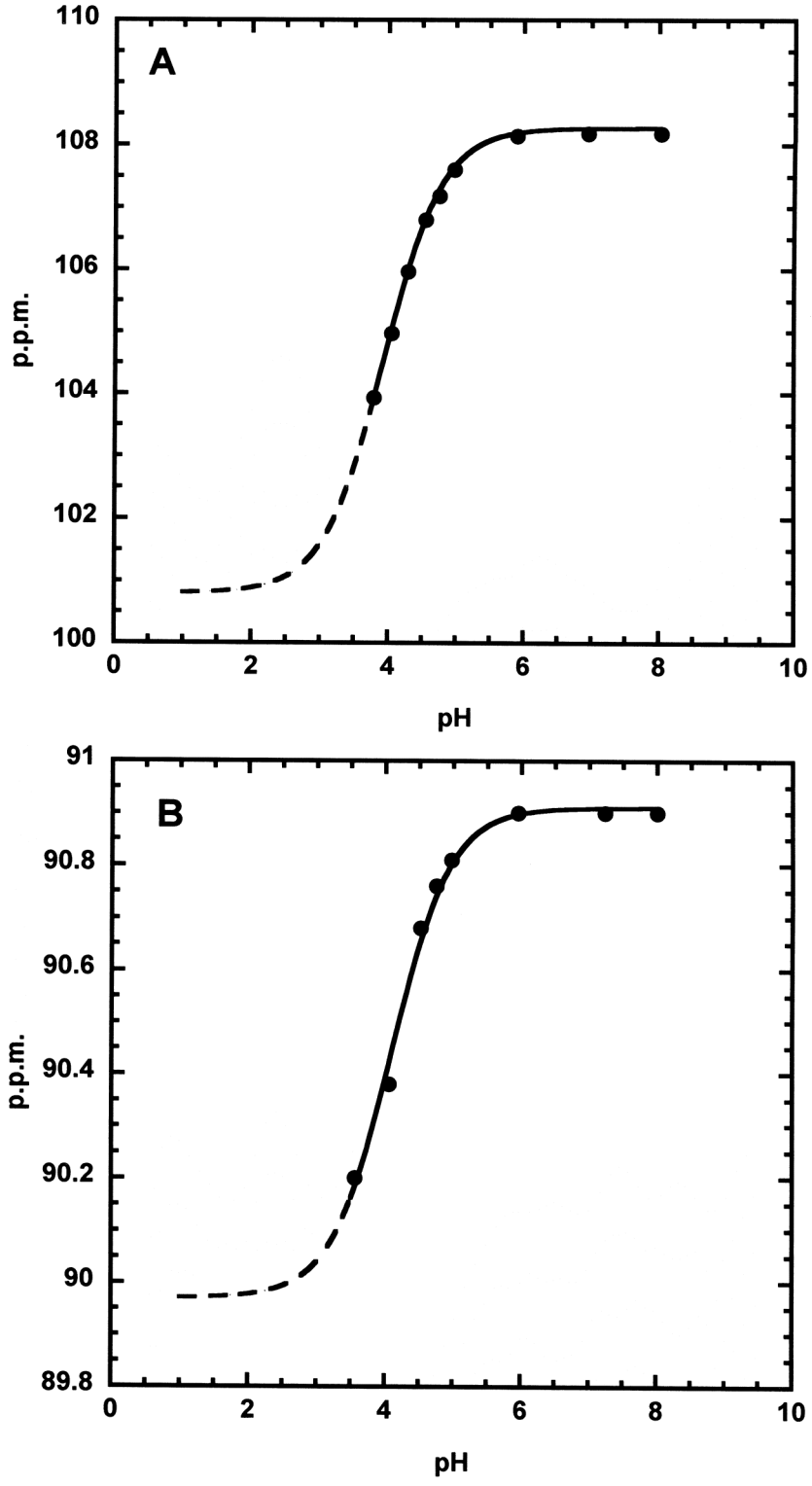

A
110
108
106
104
102
100
p.p.m.
0
2
4
6
8
10
pH
B
91
90.8
90.6
90.4
90.2
90
89.8
p.p.m.
0
2
4
6
8
10
pH

Fig5

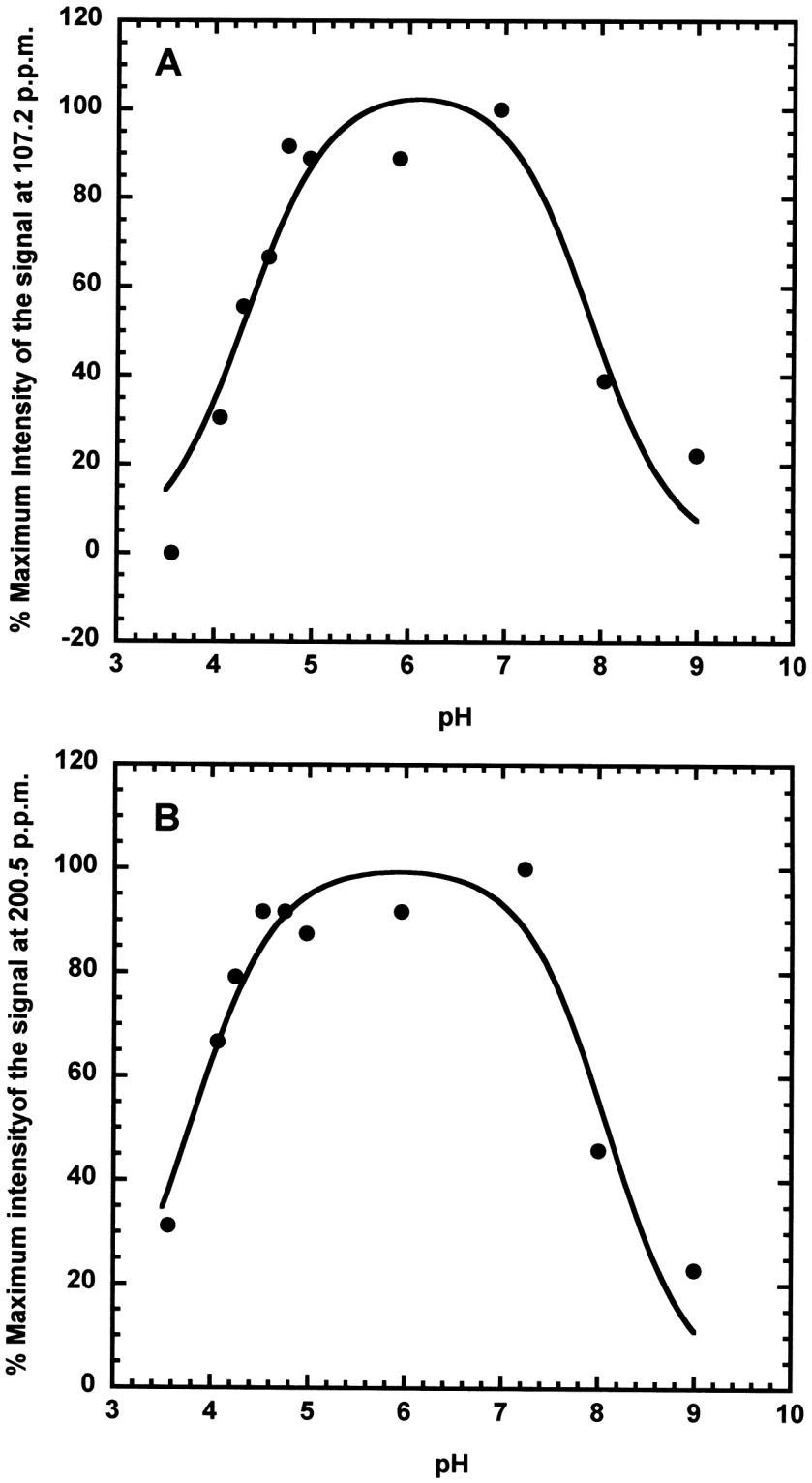
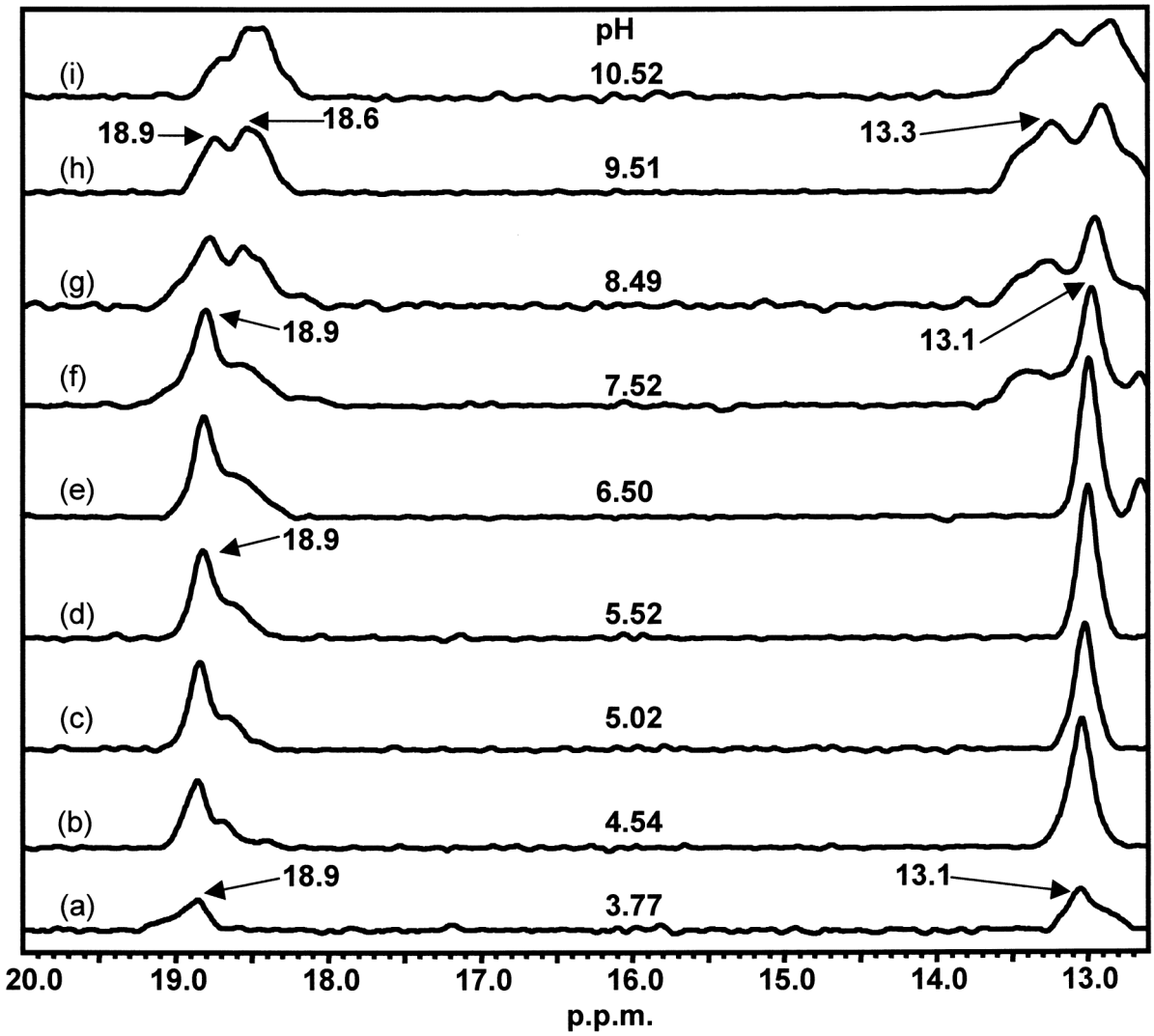
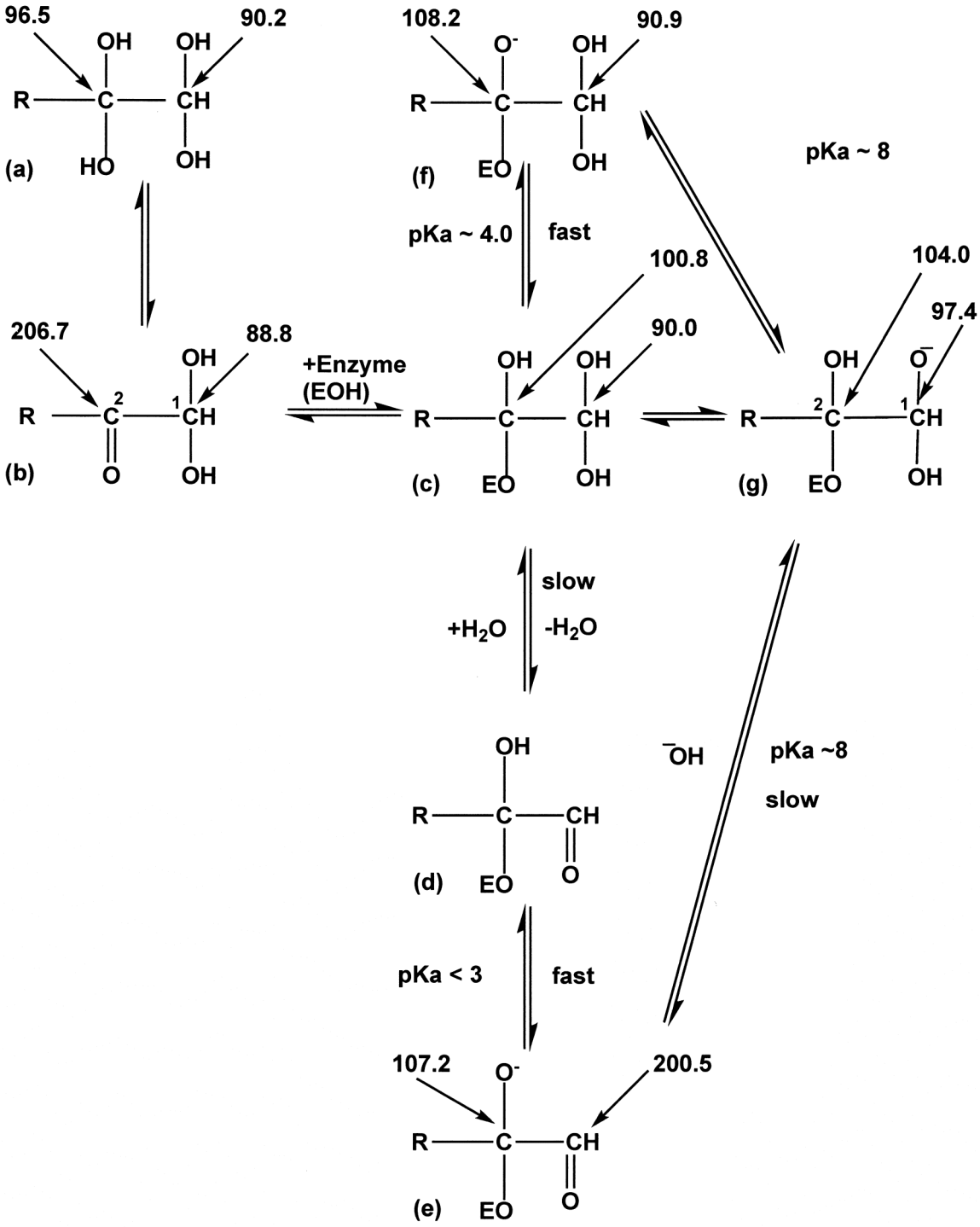

A
% Maximum Intensity of the signal at 107.2 p.p.m.
120
100
80
60
40
20
0
-20
3
4
5
6
7
8
9
10
pH
B
% Maximum intensityof the signal at 200.5 p.p.m.
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
pH

Fig6


pH
(i)
10.52
18.9
18.6
13.3
(h)
9.51
(g)
8.49
18.9
13.1
(f)
7.52
(e)
6.50
18.9
(d)
5.52
(c)
5.02
(b)
4.54
18.9
13.1
(a)
3.77
20.0
19.0
18.0
17.0
16.0
15.0
14.0
13.0
p.p.m.

Scheme1


96.5
90.2
OH
OH
R
C
CH
HO
OH
(a)
108.2
90.9
O-
OH
R
C
CH
(f)
EO
OH
pKa ~ 8
pKa ~ 4.0
fast
100.8
104.0
97.4
206.7
88.8
90.0
OH
+Enzyme
(EOH)
OH
OH
O-
R
2
C
1
CH
R
C
CH
R
2
C
1
CH
(b)
O
OH
(c)
EO
OH
(g)
EO
OH
slow
+H2O
-H2O
OH
-OH
pKa ~8
slow
R
C
CH
(d)
EO
O
pKa < 3
fast
107.2
200.5
O-
R
C
CH
(e)
EO
O

Scheme2