Title	Oxyanion and tetrahedral intermediate stabilization by subtilisin : detection of a new tetrahedral adduct
Authors(s)	Howe, Nicole, Rogers, Louis, Hewage, Chandralal, Malthouse, J.Paul G.
Publication date	2009-08
Publication information	Howe, Nicole, Louis Rogers, Chandralal Hewage, and J.Paul G. Malthouse. "Oxyanion and Tetrahedral Intermediate Stabilization by Subtilisin : Detection of a New Tetrahedral Adduct" 1794, no. 8 (August, 2009).
Publisher	Elsevier
Item record/more information	http://hdl.handle.net/10197/3022
Publisher's statement	This is the author's version of a work that was accepted for publication in Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics). Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in BBA - Proteins and Proteomics, Volume 1794, Issue 8, pages 1251-1258, DOI: 10.1016/j.bbapap.2009.04.007
Publisher's version (DOI)	10.1016/j.bbapap.2009.04.007

Downloaded 2023-04-07T10:52:29Z

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)

© Some rights reserved. For more information

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

Nicole Howe, Louis Rogers, Chandralal Hewage, J.Paul.G. Malthouse^{*}

UCD School of Biomolecular and Biomedical Science, UCD Centre for Synthesis and Chemical Biology, SEC Strategic Research Cluster, Conway Institute, University College Dublin, Dublin 4, Ireland.

^{*}Corresponding author. UCD School of Biomolecular and Biomedical Science, UCD Centre for Synthesis and Chemical Biology, SEC Strategic Research Cluster, Conway Institute, University College Dublin, Dublin 4, Ireland. Tel.: 0035317166872; fax 0035317166893
E-mail address: J.Paul.G.Malthouse@ucd.ie

Key words: protease; chymotrypsin; pH; oxyanion; subtilisin; glyoxal inhibitor.

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 ^1H -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 chloromethylketone 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 25°C 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 $[S_0] \ll K_M$. Therefore, the equation for competitive inhibition $d[P]/dt = k_{cat} * [E] * [S] / ([S] + K_M * (1 + [I] / K_i))$ reduces to $d[P]/dt = (k_{cat} / K_M) * [E] * [S] * K_i / ([I] + K_i)$. K_i 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 μ M, 50-100 μ M and 0.003-916 μ M 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

$^{\circ}\text{C}$ pulse angle; 256 transients recorded per spectrum. Waltz- 16 composite pulse ^1H 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.

^1H -NMR spectra were obtained at 500 MHz using 5 mm-diameter sample tubes. The ^1H -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 ^1H -NMR studies.

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 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) $^2\text{H}_2\text{O}$ to obtain a deuterium lock signal, as well as 10 mM potassium phosphate buffer to help maintain stable pH values during the pH titration's.

2.6. Calculation of pK_a values

The pK_a values of hemiketal hydroxyl groups were calculated using the relationship $pK_a = 15.9 - 1.42 S \sigma^*$ [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 $[S_0] \ll K_M$ 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 $0.069 \pm 0.003 \mu M$ 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 $5.32 \pm 0.34 \mu M$ at pH 7.02 has been reported [22] and in the present work a similar K_i value of $0.96 \pm 0.06 \mu M$ 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. ¹³C NMR of subtilisin inhibited by Z-Ala-Ala-[1-¹³C]Phe-glyoxal or by Z-Ala-Ala-[2-¹³C]Phe-glyoxal

Z-Ala-Ala-[2-¹³C]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 d₆-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-[2-¹³C]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-[1-¹³C]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-[1-¹³C]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-[1-¹³C]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-[2-¹³C]Phe-glyoxal and Z-Ala-Pro-[1-¹³C]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-[2-¹³C]Phe-glyoxal and Z-Ala-Ala-[1-¹³C]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-[2-¹³C]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-[2-¹³C]Phe-glyoxal. The signal at 90.9 p.p.m. in the subtilisin-Z-Ala-Ala-[1-¹³C]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. *¹H-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^{δ^1} and N^{δ^2} 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^{δ^1} proton of imidazole [30] and the presence of the signals at ~13 and ~19 p.p.m. due to the N^{δ^1} and N^{δ^2} 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 $\ll 1$. 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 K_i 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 ($\text{pK}_a \sim 4.5$) 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 $pK_a = 15.9 - 1.42 \sum \sigma^*$ [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 ($\Delta pK_a = 10.74 - 4.0$, 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 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 ($\Delta pK_a = 9.64 - 4.5 = 5.14$, Table 2) is at least 30 fold more effective in subtilisin than in chymotrypsin (Table 2).

In a recent 1H -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^H 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 (17.5 p.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 pK_a 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 pK_a 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.

Acknowledgements

This work was supported by a Research Frontiers Programme grant 05/RF/BIC026 from Science Foundation Ireland, by Basic research grant SC/2003/031 from Enterprise Ireland and by a grant from the Programme for Research in Third-Level Institutions (PRTL-3). Grant nos. 055637/Z/98 from the Wellcome Trust was used to purchase the NMR spectrometer used in these studies. We would like to acknowledge Dr Edward Spink and Mariangela Ceruso for their help in synthesising some batches of Z-Ala-Ala-[2- ^{13}C]Phe-glyoxal. We thank University College Dublin for a President's Research Fellowship for Prof. J. Paul. G. Malthouse.

References

- [1] R. Henderson, Structure of Crystalline α -Chymotrypsin, IV. The Structure of Indoleacryloyl- α -Chymotrypsin and its Relevance to the Hydrolytic Mechanism of the Enzyme, *J. Mol. Biol.* 54 (1970) 341-354.
- [2] J.D. Robertus, J. Kraut, R.A. Alden, J.J. Birktoft, Subtilisin; a Stereochemical Mechanism Involving Transition-State Stabilization, *Biochemistry* 11 (1972) 4293-4303.
- [3] J.P. Malthouse, ^{13}C - and ^1H -NMR studies of oxyanion and tetrahedral intermediate stabilization by the serine proteinases: optimizing inhibitor warhead specificity and potency by studying the inhibition of the serine proteinases by peptide-derived chloromethane and glyoxal inhibitors, *Biochem. Soc. Trans.* 35 (2007) 566-570.
- [4] R. Menard, J. Carriere, P. Laflamme, C. Plouffe, H.E. Khouri, T. Vernet, D.C. Tessier, D.Y. Thomas, A.C. Storer, Contribution of the glutamine 19 side chain to transition-state stabilization in the oxyanion hole of papain, *Biochemistry* 30 (1991) 8924-8928.
- [5] R. Menard, H.E. Khouri, C. Plouffe, R. Dupras, D. Ripoll, T. Vernet, D.C. Tessier, F. Lalberte, D.Y. Thomas, A.C. Storer, A protein engineering study of the role of aspartate 158 in the catalytic mechanism of papain, *Biochemistry* 29 (1990) 6706-6713.
- [6] R. Menard, C. Plouffe, P. Laflamme, T. Vernet, D.C. Tessier, D.Y. Thomas, A.C. Storer, Modification of the electrostatic environment is tolerated in the oxyanion hole of the cysteine protease papain, *Biochemistry* 34 (1995) 464-471.
- [7] R. Menard, A.C. Storer, Oxyanion Hole Interactions in Serine and Cysteine Proteases, *Biol. Chem. Hoppe-Seyler* 373 (1992) 393-400.

- [8] W.L. Mock, D.C.Y. Chua, Exceptional active site H-bonding in enzymes? Significance of the 'oxyanion hole' in the serine proteases from a model study, *J. Chem. Soc. Perkin Trans. 2* (1995) 2069-2074.
- [9] D. Neidhart, Y. Wei, C. Cassidy, J. Lin, W.W. Cleland, P.A. Frey, Correlation of Low-Barrier Hydrogen Bonding and Oxyanion Binding in Transition State Analogue Complexes of Chymotrypsin, *Biochemistry* 40 (2001) 2439-2447.
- [10] T.P. O'Connell, R.M. Day, E.V. Torchilin, W.W. Bachovchin, J.P.G. Malthouse, A ^{13}C -NMR study of the role of Asn-155 in stabilizing the oxyanion of a subtilisin tetrahedral adduct, *Biochem. J.* 326 (1997) 861-866.
- [11] T.P. O'Connell, J.P.G. Malthouse, A study of the stabilization of the oxyanion of tetrahedral adducts by trypsin, chymotrypsin and subtilisin, *Biochem. J.* 307 (1995) 353-359.
- [12] J.P.G. Malthouse, N.E. Mackenzie, A.S.F. Boyd, A.I. Scott, Detection of a Tetrahedral Adduct in a Trypsin-Chloromethyl Ketone Specific Inhibitor Complex by ^{13}C NMR, *J. Am. Chem. Soc.* 105 (1983) 1685-1686.
- [13] J.P.G. Malthouse, W.U. Primrose, N.E. Mackenzie, A.I. Scott, ^{13}C NMR Study of the Ionizations within a Trypsin-Chloromethyl Ketone Inhibitor Complex, *Biochemistry* 24 (1985) 3478-3487.
- [14] M.D. Finucane, E.A. Hudson, J.P.G. Malthouse, A ^{13}C -n.m.r. investigation of the ionizations within an inhibitor- α -chymotrypsin complex: Evidence that both α -chymotrypsin and trypsin stabilize a hemiketal oxyanion by similar mechanisms., *Biochem. J.* 258 (1989) 853-859.
- [15] M.D. Finucane, J.P.G. Malthouse, A study of the stabilization of tetrahedral adducts by trypsin and delta-chymotrypsin, *Biochem. J.* 286 (1992) 889-900.
- [16] D.B. O'Sullivan, T.P. O'Connell, M.M. Mahon, A. Koenig, J.J. Milne, T.P. Fitzpatrick, J.P.G. Malthouse, ^{13}C -NMR Study of How the Oxyanion pK_a Values of

- Subtilisin and Chymotrypsin Tetrahedral Adducts Are Affected by Different Amino Acid Residues Binding in Enzymes Subsites S₁-S₄, *Biochemistry* 38 (1999) 6187-6194.
- [17] T.C. Liang, R.H. Abeles, Complex of α -Chymotrypsin and N-Acetyl-L-leucyl-L-phenylalanyl Trifluoromethyl Ketone: Structural Studies with NMR Spectroscopy, *Biochemistry* 26 (1987) 7603-7608.
- [18] A. MacSweeney, G. Birrane, M.A. Walsh, T.P. O'Connell, J.P.G. Malthouse, T.M. Higgins, Crystal structure of δ -chymotrypsin bound to a peptidyl chloromethyl ketone inhibitor, *Acta Cryst. D* 56 (2000) 280-286.
- [19] E. Spink, C. Hewage, J.P. Malthouse, Determination of the structure of tetrahedral transition state analogues bound at the active site of chymotrypsin using ¹⁸O and ²H isotope shifts in the ¹³C NMR spectra of glyoxal inhibitors, *Biochemistry* 46 (2007) 12868-12874.
- [20] A. Djurdjevic-Pahl, C. Hewage, J.P.G. Malthouse, A ¹³C-NMR study of the inhibition of delta-chymotrypsin by a tripeptide-glyoxal inhibitor, *Biochem. J.* 362 (2002) 339-347.
- [21] E. Spink, S. Cosgrove, L. Rogers, C. Hewage, J.P. Malthouse, ¹³C and ¹H NMR studies of ionizations and hydrogen bonding in chymotrypsin-glyoxal inhibitor complexes, *J. Biol. Chem.* 282 (2007) 7852-7861.
- [22] A. Djurdjevic-Pahl, C. Hewage, J.P.G. Malthouse, Ionisations within a subtilisin-glyoxal inhibitor complex, *Biochim. Biophys. Acta* 1749 (2005) 33-41.
- [23] S. Cosgrove, L. Rogers, C. Hewage, J.P. Malthouse, An NMR study of the inhibition of pepsin by glyoxal inhibitors: Mechanism of tetrahedral intermediate stabilization by the aspartyl proteinases, *Biochemistry* 46 (2007) 11205-11215.

- [24] M. Liu, X. Mao, C. He, H. Huang, J.K. Nicholson, J.C. Lindon, Improved WATERGATE Pulse Sequences for Solvent Suppression in NMR Spectroscopy, *J. Magn. Reson.* 132 (1998) 125-129.
- [25] P. Ballinger, F.A. Long, Acid ionization Constants of Alcohols. II. Acidities of Some Substituted Methanols and Related Compounds, *J. Am. Chem. Soc.* 82 (1960) 795-798.
- [26] A. Dupaix, J.J. Bechet, C. Roucou, Specificity of chymotrypsin. Separation of Polar, Steric, and Specific Effects in the α -chymotrypsin-catalysed Hydrolysis of Acyl-Substituted p-Nitrophenyl esters, *Biochemistry* 12 (1973) 2559-2566.
- [27] O. Exner, Correlation analysis in Chemistry, Plenum Press, New York, 1978.
- [28] D.D. Perrin, B. Dempsey, E.P. Serjeant, pK_a Prediction for Organic Acids and Bases, Chapman and Hall, London and New York, 1981.
- [29] D. Bao, W.P. Huskey, C. Kettner, F. Jordan, Hydrogen Bonding to Active-Site Histidine in Peptidyl Boronic Acid Inhibitor Complexes of Chymotrypsin and Subtilisin: Proton Magnetic Resonance Assignments and H/D Fractionation, *J. Am. Chem. Soc.* 121 (1999) 4684-4689.
- [30] C.J. Halkides, Y.Q. Wu, C.J. Murray, A low-barrier hydrogen bond in subtilisin: ^1H and ^{15}N NMR studies with peptidyl trifluoromethyl ketones, *Biochemistry* 35 (1996) 15941-15948.
- [31] D.M. Blow, Structure and Mechanism of Chymotrypsin, *Acc. Chem. Res.* 9 (1976) 145-152.
- [32] J.D. Robertus, R.A. Alden, J.J. Birktoft, J. Kraut, J.C. Powers, P.E. Wilcox, An X-Ray Crystallographic Study of the Binding of Peptide Chloromethyl Ketone Inhibitors to Subtilisin BPN, *Biochemistry* 11 (1972) 2439-2449.

- [33] T.P. O'Connell, J.P.G. Malthouse, Determination of the ionization state of the active-site histidine in a subtilisin-(chloromethane inhibitor) derivative by ^{13}C -NMR, *Biochem. J.* 317 (1996) 35-40.
- [34] R.P. Bell, M.H. Rand, K.M.A. Wynne-Jones, Kinetics of the hydration of acetaldehyde, *Trans. Faraday Soc.* 52 (1956) 1093-1102.
- [35] D.F. De Tar, Tetrahedral Intermediate in Acyl transfer Reactions. A Revaluation of the Significance of Rate Data Used in Deriving Fundamental Linear free Energy Relationships, *J. Am. Chem. Soc.* 104 (1982) 7205-7212.
- [36] G. Bruylants, C. Redfield, K. Bartik, Developments in the characterisation of the catalytic triad of alpha-chymotrypsin: Effect of the protonation state of Asp102 on the ^1H NMR signals of His57, *Chembiochem* 8 (2007) 51-54.
- [37] P.A. Frey, Characterisation of a low barrier hydrogen bond in the active site of chymotrypsin, *J. Mol. Struct.* 615 (2002) 153-161.
- [38] J. Lin, C.S. Cassidy, P.A. Frey, Correlations of the basicity of His 57 with transition state analogue binding, substrate reactivity, and the strength of the low-barrier hydrogen bond in chymotrypsin, *Biochemistry* 37 (1998) 11940-11948.
- [39] M. Shokhen, N. Khazanov, A. Albeck, The cooperative effect between active site ionized groups and water desolvation controls the alteration of acid/base catalysis in serine proteases, *Chembiochem* 8 (2007) 1416-1421.
- [40] M. Shokhen, N. Khazanov, A. Albeck, Screening of the active site from water by the incoming ligand triggers catalysis and inhibition in serine proteases, *Proteins* 70 (2008) 1578-1587.
- [41] A. Warshel, G. Naray-Szabo, F. Sussman, J.K. Hwang, How Do Serine Proteases Really Work?, *Biochemistry* 28 (1989) 3629-3637.

- [42] M. Komiyama, M.L. Bender, Do cleavages of amides by serine proteases occur through a stepwise pathway involving tetrahedral intermediates?, *Proc. Natl. Acad. Sci. U.S.A.* 76 (1979) 557-560.

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) $^2\text{H}_2\text{O}$ 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) $^2\text{H}_2\text{O}$ 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) $^2\text{H}_2\text{O}$.

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) $^2\text{H}_2\text{O}$ 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) $^2\text{H}_2\text{O}$.

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.

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

The fitted parameters were (a) Subtilisin and Z-Ala-Ala-[2-¹³C]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-[1-¹³C]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 ¹³C-NMR signals at 107.2 p.p.m. and 200.5 p.p.m. in the complexes formed between subtilisin and Z-Ala-Ala-[2-¹³C]Phe Glyoxal and Z-Ala-Ala-[1-¹³C]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.

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

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

Fig. 6. Effect of pH on the ¹H-NMR signals from Z-Ala-Pro-[2-¹³C]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-[2-¹³C]Phe Glyoxal containing 1.00 mM subtilisin and 1.64% (v/v) d₆-dimethyl sulfoxide at pH = 6.50. The volumes of 1M HCl containing 10% (v/v) ²H₂O 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-[2-¹³C]Phe Glyoxal containing 1.14 mM subtilisin and 1.60 % (v/v) d₆-dimethyl sulfoxide at pH = 7.52. The volumes of 1M KOH containing 10% (v/v) ²H₂O 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	$K_{d(\text{obs})}$ (μM)	pH	$K_{d(\text{obs})}$ (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

Fig2

Fig3

Fig4

Fig5

Fig6

Scheme 1

Scheme2