A ^{13}C -NMR study of the role of Asn-155 in stabilizing the oxyanion of a subtilisin tetrahedral adduct

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By removing one of the hydrogen-bond donors in the oxyanion hole of subtilisin BPN, we have been able to determine how it affects the catalytic efficiency of the enzyme and the pK_a of the oxyanion formed in a chloromethane inhibitor derivative. Variant 8397 of subtilisin BPN contains five mutations which enhance its stability. Site-directed mutagenesis was used to prepare the N155A mutant of this variant. The catalytic efficiencies of wild-type and variant 8397 are similar, but replacing Asn-155 with alanine reduces catalytic efficiency approx. 300-fold. All three forms of subtilisin were alkylated using benzyl-oxy-carbonyl-glycyl-glycyl[2- ^{13}C]phenylalanylchloromethane and examined by ^{13}C -NMR. A single signal due to the ^{13}C -enriched

carbon was detected in all the derivatives and it was assigned to the hemiketal carbon of a tetrahedral adduct formed between the hydroxy group of Ser-221 and the inhibitor. This signal had chemical shifts in the range 98.3–103.6 p.p.m., depending on the pH. The titration shift of 4.7–4.8 p.p.m. was assigned to oxyanion formation. The oxyanion pK_a values in the wild-type and 8397 variants were 6.92 and 7.00 respectively. In the N155A mutant of the 8397 variant the oxyanion pK_a increased to 8.09. We explain why such a small increase is observed and we conclude that it is the interaction between the oxyanion and the imidazolium cation of the active-site histidine that is the main factor responsible for lowering the oxyanion pK_a .

INTRODUCTION

Catalysis by the serine proteinases proceeds via an acyl intermediate. Both the formation and breakdown of this acyl intermediate is thought to occur via a tetrahedral intermediate which is formed by the addition of the hydroxy group of the active-site serine residue to the substrate carbonyl about to be hydrolysed. Henderson [1] was the first to postulate that in chymotrypsin the carbonyl oxygen of the substrate might be hydrogen-bonded to the backbone NH groups of Gly-193 and Ser-195 in chymotrypsin. He also suggested that these hydrogen bonds to the carbonyl oxygen might be stronger in the tetrahedral intermediate, although he did point out that his X-ray-crystallographic data were not accurate enough to confirm this. This idea was adopted by Robertus et al. [2], who proposed that, in subtilisin, the backbone NH of Ser-221 and the side-chain NH_2 group of Asn-155 hydrogen-bonded to the oxyanion of the tetrahedral intermediate. They were the first to suggest that the oxyanion-binding site should be called the oxyanion hole. Subsequently an oxyanion hole has been found at the active site of a range of proteolytic enzymes [3–5]. Studies of a range of tetrahedral adducts that mimic the structure of a tetrahedral intermediate have shown that the oxyanion is positioned in the oxyanion hole such that it will form hydrogen bonds to the enzyme [3]. X-ray-crystallographic studies [6] and NMR studies [7] with aldehyde inhibitors has shown that the oxyanion can either be in the oxyanion hole or point out of the oxyanion hole towards the active-site histidine. However, with chloromethane inhibitor derivatives of subtilisin [8], thrombin [9] and protease B of *Streptomyces griseus* [10] the oxyanion is found in the oxyanion hole.

Structural studies do not tell us whether these hydrogen bonds are required for efficient catalysis. Kinetic studies using thiono and oxygen ester substrates suggest that hydrogen-bonding in the oxyanion hole is essential for efficient catalysis by the serine

proteases but not for the thiol proteases [11]. An alternative approach for determining the importance of hydrogen-bonding to the oxyanion is to remove hydrogen-bonding groups by site-directed mutagenesis. For serine proteases such as chymotrypsin and trypsin, this approach cannot be used because the hydrogen-bonding groups are backbone NH groups. However, in subtilisin only one hydrogen-bonding group is a backbone NH group (from Ser-221); the other hydrogen bonding groups are an NH_2 group and an hydroxy group from Asn-155 and Thr-220 respectively. Therefore site-directed mutagenesis has been used to determine how hydrogen-bonding from the oxyanion to Asn-155 [12] and Thr-220 [13] contributes to catalytic efficiency in subtilisin. These studies showed that hydrogen-bonding to the oxyanion made a significant contribution to catalytic efficiency.

There have been numerous attempts to explain how hydrogen-bonding to the oxyanion could enhance catalytic efficiency. Henderson [1] suggested that it might be stronger for a tetrahedral intermediate, so lowering its free energy and favouring its formation. Resonance Raman spectroscopy of acyl intermediates has led to the suggestion that hydrogen-bonding of a carbonyl group in the oxyanion hole may enhance catalysis by causing distortion of the sp^2 -hybridized carbonyl carbon towards an sp^3 -hybridized tetrahedral state [14]. Curved proton inventory experiments [15] with methyl chymotrypsin were explained by suggesting strengthened oxyanion hydrogen-bonding, which appears to support this proposal. However, it has recently been shown that this result was due to a medium effect and not to oxyanion hydrogen-bonding [16]. It has also been suggested that short very strong low-barrier hydrogen bonds are used to stabilize the oxyanion [17]. But several workers have argued that, as the hydrogen-bond donors and oxyanion do not have similar pK_a values, there will not be low-barrier hydrogen-bond formation [18]. Another proposal is that, if the hydrogen-bonding groups to the oxyanion act as pre-aligned dipoles, they would stabilize the oxyanion more effectively than in water, as solvent reorganization

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is avoided [19,20]. However, it has been argued that there is no experimental evidence that pre-aligned dipoles make a significant contribution to oxyanion stabilization in subtilisin [21] and that it is not likely that they would make a significant contribution to oxyanion stabilization in subtilisin, as the side chain of Asn-155 which acts as a hydrogen-bond donor is not fixed in position [22]. In this work we use ^{13}C -NMR and site-directed mutagenesis to study oxyanion stabilization in a subtilisin chloromethane derivative.

Specific substrate-derived chloromethane inhibitors of trypsin [23,24], chymotrypsin [25,26] and subtilisin [27–29] alkylate N-3 of the active-site histidine residue. ^{13}C -NMR has been used to show that, in the trypsin [30–32], chymotrypsin [21,33] and subtilisin [22] derivatives, the active-site serine hydroxy group adds to the ketone carbon of the inhibitor to form a tetrahedral adduct analogous to the tetrahedral intermediate thought to be formed during catalysis. The pH-dependent changes of the chemical shift of the hemiketal [21,22,31,33] and α -methylene carbons [21,34,35] have been used to determine the oxyanion and imidazolium pK_a values respectively. A recent study using ^{15}N and ^1H NMR has confirmed the titration behaviour of the imidazolium ion in trypsin and chymotrypsin chloromethane derivatives [36]. It has also been shown that the chloromethane derivatives of trypsin [31,34], chymotrypsin [21] and subtilisin [35] all stabilize zwitterionic tetrahedral adducts analogous to a catalytic tetrahedral intermediate. They do this by raising the pK_a of the imidazolium cation of the active-site histidine residue while lowering the pK_a of the oxyanion. This has led to the proposal that for optimal catalytic efficiency these enzymes must stabilize zwitterionic tetrahedral intermediates and so they have all evolved to stabilize these intermediates [21,31,33,35]. The decrease in the oxyanion pK_a could be due to its hydrogen-bonding interactions in the oxyanion hole and/or to an electrostatic interaction with the imidazolium cation of the active-site histidine residue. In the present work we attempt to answer this question by determining how removing the hydrogen bond from the side-chain NH_2 of Asn-155 affects the oxyanion pK_a in subtilisin chloromethane inhibitor derivatives.

MATERIALS AND METHODS

The materials and methods used for preparing and analysing samples of alkylated subtilisin for ^{13}C -NMR spectroscopy were as described previously [22].

Preparation of the 8397 variant of subtilisin BPN and the N155A mutant of the 8397 variant of subtilisin BPN

The pGx8397 vector containing the *apr* gene was a gift from Genex Corp. This plasmid encodes subtilisin BPN containing five mutations: M50F, N76D, G169A, Q206C and N218S, which stabilize the 8397 variant of subtilisin BPN at high pH [37,38]. The *apr* gene was subcloned into the pBluescript KS⁺ vector (Stratagene) using *Bam*HI–*Sal*I restriction sites, and the N155A mutation of the 8397 variant of subtilisin BPN was introduced in the 8397 variant of subtilisin BPN gene by thermal cycling (PCR). Oligonucleotides were made that spanned the vector T7 and reverse primer sites respectively; 5'-CGA CTC ACT ATA GGG CGA ATT GGG TAC-3' and 5'-ACA GCT ATG ACC ATG ATT ACG CCA AGC-3'. Oligonucleotides were also made encoding the N155A mutation complementary to both the coding and non-coding strands of the BPN gene respectively: 5'-A AGT GGC TTC AGC ACC GGC TGC CGC AAC G-3', and 5'-G GCA GCC GGT GCT GGA GGC ACT TCC GGC A-3' (mutagenic codons are underlined). Reactions were performed with vent DNA polymerase (New England Biolabs) using the

overlap extension method [39]. The product was cleaved with appropriate restriction enzymes and ligated back into the pGx83 vector. The N155A mutation was confirmed by DNA sequencing. The rest of the protein sequence was verified by sequencing and amino acid analysis of the purified protein.

Isolation of the 8397 variant of subtilisin BPN and the N155A mutant of the 8397 variant of subtilisin BPN

Isolation procedure 1

The 8397 variant of subtilisin BPN was produced from genetically transformed *Bacillus subtilis* DB104 strain (*his*[−], *apr*[−], *npr*[−]) [40]. Bacteria producing the 8397 variant of subtilisin BPN were grown for 14–16 h at 37 °C in modified S7 medium [41]. Cells were pelleted by centrifugation at 15000 *g* for 1 h at 4 °C. The supernatant (1 litre) containing the 8397 variant of subtilisin BPN was dialysed overnight against 30 litres of 10 mM tris(hydroxymethyl)aminomethane/HCl at pH 6.0 and at 4 °C. The protein was concentrated to approx. 50 ml by ultrafiltration using an Amicon PM10 membrane. It was then dialysed overnight against 2.5 litres of 0.1 M NaCl/20 mM CaCl_2 at pH 6.2. Just before examination by NMR, this eluate was concentrated on an Amicon PM10 membrane to a volume of approx. 30 ml. It was then dialysed against 2 × 1 litre of 10 mM tris(hydroxymethyl)aminomethane/HCl buffer at pH 7.5 before concentrating to a volume of approx. 1 ml for NMR spectroscopy.

When the wild-type and N155A mutant of the 8397 variant of subtilisin BPN were analysed by SDS/PAGE an intense band of protein of M_r approx. 27000 was detected with only trace amounts of other proteins being observed.

Isolation procedure 2

The supernatant (1 litre) containing the 8397 variant of subtilisin BPN was prepared as described above. It was then dialysed overnight against 25 litres of 1 mM sodium phosphate at pH 5.8 and 4 °C. The dialysed protein was passed down a column (2.5 cm × 20 cm) of Whatman CM52 CM-cellulose ion exchanger equilibrated with 1 mM sodium phosphate, pH 5.8, and the protein was eluted from the column with a solution containing 0.1 M NaCl and 1 mM sodium phosphate at pH 6.0. The eluate (approx. 150 ml) was dialysed against 5 litres of 0.1 M NaCl/20 mM CaCl_2 at pH 6.2.

Just before alkylation, this eluate was concentrated on an Amicon PM10 membrane to a volume of approx. 30 ml. It was dialysed against 2 × 1 litre of 10 mM tris(hydroxymethyl)aminomethane/HCl buffer at pH 7.5, concentrated to 15 ml, and then 15 ml of 0.2 M potassium phosphate at pH 7 was added.

When the wild-type and N155A mutant of the 8397 variant of subtilisin BPN were analysed by SDS/PAGE, a single intense band of protein of M_r approx. 27000 was detected. Titration using *N-trans*-cinnamoylimidazole [22] showed that 71 % of the protein of the wild-type 8397 variant of subtilisin was active. With the wild-type 8397 variant of subtilisin some autolysis products were detected when the samples aged. Owing to the lower proteolytic activity of N155A mutant subtilisin BPN, autolysis products were not detected in samples of this enzyme.

Alkylation of the 8397 variant of subtilisin BPN and the N155A mutant of the 8397 variant of subtilisin BPN by Z-Gly-Gly-[2- ^{13}C]Phe-CH₂Cl

Samples of the 8397 variant of subtilisin BPN were alkylated with Z-Gly-Gly-[2- ^{13}C]Phe-CH₂Cl as described previously [22]. Because of the lower reactivity of the N155A mutant of the 8397 variant of subtilisin BPN towards Z-Gly-Gly-[2- ^{13}C]Phe-CH₂Cl,

a modified alkylation procedure was used with this mutant form of subtilisin BPN.

Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl (20 mM) was dissolved in 1,4-dioxan and portions were added to 30 ml of 0.06 mM N155A-subtilisin in 5 mM tris(hydroxymethyl)aminomethane/HCl and 0.1 M phosphate buffer at pH 7.0, over a period of 3 days to give a total concentration of 0.36 mM Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl. The reaction mixture was gently stirred and the extent of inhibition was determined by assaying with *N*-3-carboxypropionyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide as described by Zhong et al. [42]. After alkylation, only 5% of the original catalytic activity remained. The solution was centrifuged at 15600 *g* and the supernatant was concentrated to a volume of approx. 5 ml by ultrafiltration on an Amicon PM10 membrane. It was then dialysed against 3 $\times$ 100 ml of 5 mM potassium phosphate buffer, pH 7.0, to remove low- M_r materials. The alkylated N155A mutant of the 8397 variant of subtilisin BPN was then concentrated by ultrafiltration on an Amicon PM10 membrane.

Assay of enzyme solutions

Subtilisin solutions were assayed as described by O'Connell and Malthouse [22]. It was assumed that the 8397 variant of subtilisin BPN and the N155A mutant of the 8397 variant of subtilisin BPN had the same ϵ_{280} (32300 M $^{-1}$ ·cm $^{-1}$) as commercial subtilisin BPN [43].

NMR spectroscopy

After alkylation with Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl it was assumed that for both the 8397 variant of subtilisin BPN and the N155A mutant of this variant the spin lattice relaxation times of the ^{13}C -enriched hemiketal carbon atoms would be 0.48 s, which is the same value as that of the same carbon atom in alkylated commercial subtilisin BPN [22]. NMR spectra were recorded at 1.88 T with a Bruker WP80 wide-bore spectrometer operating at 20.115 MHz for ^{13}C nuclei as described previously [22].

Determination of the catalytic parameters for the subtilisin BPN-catalysed hydrolysis of *N*-3-carboxypropionyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide

Commercial subtilisin BPN was used to catalyse the complete hydrolysis of *N*-3-carboxypropionyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide at pH 8.2. The amount of *p*-nitroaniline released was determined using ϵ_{410} 8800 M $^{-1}$ ·cm $^{-1}$ [44] and from this it was calculated that ϵ_{315} was 13300 M $^{-1}$ ·cm $^{-1}$ for the substrate. This absorption coefficient was used to determine substrate concentrations.

Steady-state parameters were obtained at 25 °C in 0.1 M tris(hydroxymethyl)aminomethane/HCl buffer containing 3% (v/v) DMSO at pH 8.2 [42]. Michaelis parameters were determined from initial-rate data by computer fitting to the hyperbolic Michaelis-Menten equation. For commercial subtilisin BPN and for the 8397 variant of subtilisin BPN, the amount of active enzyme was determined by titration with *N*-*trans*-cinnamoylimidazole [22]. Some 62% and 71% of the protein of the commercial and 8397 variant of subtilisin BPN were fully active protein by this method. However, because of the low reactivity of the N155A mutant of the 8397 variant of subtilisin towards *N*-*trans*-cinnamoylimidazole, the active enzyme concentration could not be determined using this active-site titrant. Therefore we assumed that all the protein of the N155A mutant of the 8397 variant of subtilisin BPN was fully active. This we believe is a reasonable assumption as the N155A

mutant of the 8397 variant of subtilisin BPN has a very low catalytic activity and so it is not expected to undergo significant autolysis.

RESULTS AND DISCUSSION

Alkylation of wild-type and mutant subtilisin BPN

After alkylation of commercial subtilisin BPN by Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl [22] one signal was detected (signal B, Figure 1, spectrum b) which was not present in the ^{13}C -NMR spectrum of commercial subtilisin BPN (Figure 1, spectrum a). The signals at

Figure 1 ^{13}C -NMR spectra of wild-type subtilisin and of the N155A mutant of variant 8397 of subtilisin before and after alkylation by Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl

Acquisition parameters were as described in the Materials and methods section. An exponential weighting factor of 5 Hz was used for all spectra. The number of transients recorded per spectrum were: 49152 for spectra (a) and (b); 73728 for spectrum (c); 55336 for spectra (d), (e) and (f); 113400 for spectrum (g); 16200 for spectrum (h); 64800 for spectrum (i). Spectrum (a) is of wild-type subtilisin, spectrum (b) is of the Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin derivative prepared from wild-type subtilisin, spectrum (c) is of mutant N155A of subtilisin variant 8397 isolated by procedure 1 as described in the Materials and methods section, spectra (d)–(f) are of mutant N155A of subtilisin variant 8397 isolated by procedure 2 as described in the Materials and methods section, and spectra (g)–(i) are of the Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin derivative prepared from mutant N155A of subtilisin variant 8397 isolated by procedure 2 as described in the Materials and methods section. Sample conditions were: (a) 1.0 ml of 1.8 mM subtilisin at pH 7.0; (b) 1.0 ml of 2.0 mM Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin at pH 7.0; (c) 1.0 ml of 1.8 mM subtilisin at pH 7.0; (d) 1.0 ml of 1.6 mM subtilisin at pH 7.1; (e) 1.1 ml of 1.4 mM subtilisin at pH 4.6; (f) 1.2 ml of 1.3 mM subtilisin at pH 10.3; (g) 1.0 ml of 1.6 mM Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin at pH 7.2; (h) 1.1 ml of 1.6 mM Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin at pH 8.9; (i) 1.6 ml of 1.2 mM Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin at pH 10.3.

Figure 2 pH titration of the signals due to the ^{13}C -enriched carbon atoms in Z-Gly-Gly-[2- ^{13}C]-CH $_2$ -subtilisin BPN' variant 8397 and Z-Gly-Gly-[2- ^{13}C]-CH $_2$ -A155N-subtilisin BPN' variant 8397

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

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

Line A (●) was calculated for Z-Gly-Gly-[2- ^{13}C]-CH $_2$ -subtilisin BPN' variant 8397 using the fitted parameters $\text{p}K_a = 7.00 \pm 0.02$, $S_1 = 98.75 \pm 0.04$ and $S_2 = 103.56 \pm 0.03$. Line B (■) was calculated for Z-Gly-Gly-[2- ^{13}C]-CH $_2$ -A155N-subtilisin BPN' variant 8397 using the fitted parameters $\text{p}K_a = 8.09 \pm 0.03$, $S_1 = 98.26 \pm 0.05$ and $S_2 = 103.00 \pm 0.04$.

41.5–45.0 p.p.m. in Figure 1, spectra b–i were due to 50 mM glycine and 50 mM glycylglycine added to maintain stable pH values [22,35]. However, when the unmodified N155A mutant of the 8397 variant of subtilisin BPN was examined by ^{13}C -NMR, it was found to contain additional signals (Figure 1, spectrum c) which were not due to these buffers and they were not present in commercial subtilisin BPN (Figure 1, spectrum a). This sample of the N155A mutant of subtilisin 8397 was obtained by dialysing and concentrating the appropriate growth medium as described in isolation procedure 1 in the Materials and methods section. We believe that these additional ^{13}C -NMR signals (at 22.8 p.p.m. and 61.4–104.9 p.p.m. in Figure 1, spectrum c) arise from a high- M_r carbohydrate which has been produced by *Bacillus subtilis* and which has co-purified with the enzyme. This was confirmed by the fact that only protein samples containing these signals gave a positive reaction with phloroglucinol which is specific for pentoses, glycuronic acid, galactose and galactans [45]. The negative reaction obtained with Fehling's and Benedict's solutions is expected if the carbohydrate is not a reducing sugar or if it is highly polymerized [45]. The intensity of the additional ^{13}C -NMR signals were reduced relative to the protein signals (Figure 1, spectrum d) by introducing an additional purification step in which the enzyme was passed through a CM-cellulose ion

exchanger as described in isolation procedure 2 in the Materials and methods section. The signal at 98.5 p.p.m. (signal A, Figure 1, spectrum d) is characteristic of the anomeric C-1 carbons of a range of carbohydrates [46,47]. The chemical shift of signal A did not change when the pH was lowered from 7 (Figure 1, spectrum d) to 4.6 (Figure 1, spectrum e) or raised to 10.3 (Figure 1, spectrum f) which shows that $\text{p}K_a \geq 12$, as is expected for the anomeric carbon of a sugar [48]. However, after alkylation of the N155A mutant of subtilisin 8397, a new signal (signal B) at 98.7 p.p.m. was detected which masked the smaller signal (signal A) at 98.5 p.p.m. due to carbohydrate (Figure 1, spectrum g). On raising the pH to 8.9 (Figure 1, spectrum h) and 10.3 (Figure 1, spectrum i) the chemical shift of signal B increased and signal A at 98.5 p.p.m. was revealed.

Comparison of the properties of the ^{13}C -enriched carbon of wild-type and variant 8397 forms of Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin BPN

The ^{13}C -enriched carbon of Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ -subtilisin BPN variant 8397 titrated from 98.75 to 103.56 p.p.m. as the pH increased (Figure 2). This titration shift of 4.81 p.p.m. was due to a single ionizing group with a $\text{p}K_a$ of 7.00 (Figure 2). The titration shift, $\text{p}K_a$ value and chemical-shift values for this variant were essentially the same as those previously obtained [22] for the same derivative formed with wild-type subtilisin (see Table 2). The signals are assigned to a hemiketal formed by the addition of the hydroxy group of Ser-221 to the ^{13}C -enriched inhibitor carbonyl carbon (structures A and B in Scheme 1), with the titration shift resulting from deprotonation of the hemiketal hydroxy group (structure A in Scheme 1) to form an oxyanion (structure B in Scheme 1) as in earlier studies [21,22]. Therefore we conclude that the five mutations (M50F, N76D, G169A, Q206C and N218S) of the 8397 variant of subtilisin BPN do not have a significant effect on the $\text{p}K_a$ of the oxyanion in this inhibitor derivative.

Effect of mutating Asp-155 to Ala-155 on the subtilisin-catalysed hydrolysis of *N*-3-carboxypropionyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide

Replacing Asn-155 with an alanine residue leads to an approx. 200–300-fold decrease in k_{cat} and k_{cat}/K_m when *N*-3-carboxypropionyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide is used as substrate (Table 1). This decrease in k_{cat}/K_m suggests that the hydrogen bond from the side chain of Asn-155 contributes 14.1 kJ/mol to transition-state stabilization. A similar value of 17.6 kJ/mol has been estimated when this mutation was carried out with an S24C variant of subtilisin [13] and values of 12, 13, 14, 15 and 20 kJ/mol were also estimated when Asn-155 was replaced by Gly, Gln, Leu, Asp and Thr respectively [12,49,50]. It has been shown that the deletion of a group on an enzyme or substrate that should form a hydrogen bond with a charged group affects binding by approx. 16.7 kJ/mol or by a factor of approx. 1000 [51]. Therefore these results are consistent with the suggestion that hydrogen-bonding from the oxyanion to the side chain of Asn-155 stabilizes the tetrahedral intermediate and that formation or breakdown of the tetrahedral intermediate is rate-limiting with this substrate.

Effect of mutating Asp-155 to Ala-155 on the oxyanion $\text{p}K_a$ of the Z-Gly-Gly-[2- ^{13}C]Phe-CH $_2$ Cl derivative of the 8387 variant of subtilisin BPN

After this mutation the titration behaviour of the hemiketal carbon (line B, Figure 2) was very similar to that of the control

Scheme 1 Structure and chemical shifts of wild-type and mutant forms of Z-Gly-Gly-[2-¹³C]-CH₂-subtilisin

Table 1 Catalytic parameters for the hydrolysis of *N*-3-carboxypropionyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide by wild-type and mutant subtilisins at pH 8.2

Enzyme	Variant	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)	K_m (mM)	Reference
Subtilisin	Wild-type	51.0	282 000	0.181	Present work
Subtilisin	Wild-type	47.0	270 000	0.172	[38]
Subtilisin	8397	81.9	1270 000	0.0647	Present work
Subtilisin	8397	74.0	760 000	0.097	[38]
N155A-subtilisin	8397	0.44	4360	0.101	Present work

Table 2 Titration constants obtained by ¹³C-NMR studies of wild-type and mutant subtilisins inhibited by Z-Gly-Gly-[2-¹³C]Phe-CH₂Cl

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

Enzyme	Variant	Inhibitor	Chemical shift (p.p.m.)			
			δ_1	δ_2	$\delta_2 - \delta_1$	pK _a
Subtilisin	Wild-type	Z-Gly-Gly-[2- ¹³ C]Phe-CH ₂ Cl	98.87	103.59	4.72	6.92
Subtilisin	8397	Z-Gly-Gly-[2- ¹³ C]Phe-CH ₂ Cl	98.75	103.56	4.81	7.00
N155A-subtilisin	8397	Z-Gly-Gly-[2- ¹³ C]Phe-CH ₂ Cl	98.27	103.00	4.73	8.09

derivative formed with the 8397 variant (line A, Figure 2), except that the oxyanion pK_a increased by 1.09 pK_a units (Table 2). This pK_a increase corresponds to a free-energy change of 6.2 kJ/mol.

In order to lower the pK_a of the oxyanion, the hydrogen bond from Asn-155 should either stabilize the oxyanion or destabilize its conjugate acid. The free energies of uncharged hydrogen bonds and hydrogen bonds involving one charged group and one neutral group have been estimated [51,52]. The hydrogen bond between the negatively charged oxyanion and Asn-155 should have a free energy of 12.6–25.2 kJ/mol, whereas the free energy of the hydrogen bond between the neutral conjugate acid of the oxyanion and Asn-155 is expected to have a free energy of 2.1–7.5 kJ/mol [51,52]. Therefore we estimate that hydrogen-bonding to Asn-155 should preferentially stabilize the oxyanion by 5.1–23.1 kJ/mol which should lower the oxyanion pK_a by 0.9–4.0 pK_a units. The observed increase of 1.09 pK_a units in the oxyanion pK_a when Asn-155 is replaced by alanine in the chloromethane derivative formed with the N155A mutant of the subtilisin-8397 variant is within the lower part of this range. The small change in pK_a suggests that there is a low charge density on the oxyanion in subtilisin chloromethane derivatives. This conclusion is supported by the low pK_a of the oxyanion in the wild-type subtilisin chloromethane derivatives.

The oxyanion and its conjugate acid are approx. 3.0 Å from the nitrogen of the backbone NH of Ser-221 and Nδ2 of the side chain of Asn-155. If the oxyanion in subtilisin is not stabilized by hydrogen bonds acting as pre-aligned dipoles [21,22], then the energetic contribution of multiple hydrogen bonds is expected to be essentially additive [53]. If also it is assumed that both these hydrogen bonds are equivalent, then we would expect that hydrogen-bonding of the oxyanion to the backbone NH of Ser-221 would contribute approx. 14.1 kJ/mol to tetrahedral intermediate stabilization and decrease the oxyanion pK_a by approx. 1.09 pK_a units (Δ*G* approx. 6.2 kJ/mol). However, O_γ of Thr-220 is approx. 4.6 Å from the oxyanion and has been shown to

contribute only approx. 7.5 kJ mol to tetrahedral intermediate stabilization [13]. Therefore we would expect this hydrogen bond to decrease the oxyanion pK_a by only about 0.58 pK_a unit ($\Delta G \approx 3.3$ kJ/mol). Thus, although hydrogen-bonding in the oxyanion hole is expected to stabilize catalytic tetrahedral intermediates by about 35.7 kJ/mol, it is only expected to contribute approx. 15.7 kJ/mol to lowering the oxyanion pK_a in chloromethane derivatives. Consequently hydrogen-bonding in the oxyanion hole should only lower the oxyanion pK_a by about 2.8 pK_a units. But only two of the three hydrogen bonds to the oxyanion are optimal and so a similar or larger pK_a decrease would be expected from an equivalent number of hydrogen bonds in water. This would apply for both catalytic tetrahedral intermediates and tetrahedral adducts in subtilisin chloromethane inhibitor derivatives. But we have estimated that in subtilisin chloromethane derivatives the oxyanion pK_a is lowered by 5 pK_a units relative to its value in water [22]. Therefore this decrease of 5 pK_a units is attributed to the interaction of the oxyanion with the imidazolium cation of the active-site histidine. We conclude that in subtilisin chloromethane derivatives the hydrogen bond from the side chain of Asn-155 to the oxyanion plays a significant but minor role in lowering the pK_a of the oxyanion. Recent model studies with salicylamides support this suggestion, as they have calculated that an intramolecular hydrogen bond involving an amide NH group and an oxyanion will only produce a small decrease in the oxyanion pK_a [54]. These conclusions are in agreement with earlier proposals that the decreases in oxyanion pK_a values in subtilisin, trypsin and chymotrypsin chloromethane derivatives are largely due to their interaction with the imidazolium cation of the active-site histidine and that the main role of hydrogen-bonding by the oxyanion is to provide localized solvation of the oxyanion [21,22,30,31,33].

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