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# **Mechanism of the binding of Z-L-tryptophan and Z-L-phenylalanine to thermolysin and stromelysin-1 in aqueous solutions**

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## ABSTRACT

The chemical shift of the carboxylate carbon of Z-tryptophan is increased from 179.85 to 182.82 ppm and 182.87 on binding to thermolysin and stromelysin-1 respectively. The chemical shift of Z-phenylalanine is also increased from 179.5 ppm to 182.9 ppm on binding to thermolysin. From pH studies we conclude that the pK<sub>a</sub> of the inhibitor carboxylate group is lowered by at least 1.5 pK<sub>a</sub> units when it binds to either enzyme. The signal at ~183 ppm is no longer observed when the active site zinc atom of thermolysin or stromelysin-1 is replaced by cobalt. We estimate that the distance of carboxylate carbon of Z-[1-<sup>13</sup>C]-L-tryptophan is  $\leq 3.71 \text{ \AA}$  from the active site cobalt atom of thermolysin. We conclude that the side chain of Z-[1-<sup>13</sup>C]-L-tryptophan is not bound in the S<sub>2</sub>' subsite of thermolysin. As the chemical shifts of the carboxylate carbons of the bound inhibitors are all ~183 ppm we conclude that they are all bound in a similar way most probably with the inhibitor carboxylate group directly coordinated to the active site zinc atom. Our spectrophotometric results confirm that the active site zinc atom is tetrahedrally coordinated when the inhibitors Z-tryptophan or Z-phenylalanine are bound to thermolysin.

**Key words:** thermolysin; stromelysin-1; NMR; amino acids, binding, cobalt

**Abbreviations:** MCA, (7-methoxycoumarin-4-yl)acetyl; stromelysin-1, stromelysin-1 catalytic domain<sup>83-247</sup>.

## 1. Introduction

Thermolysin is a bacterial zinc metalloproteinase. It is an endoprotease and has been extensively studied and shown to preferentially catalyse the hydrolysis of peptide bonds involving the  $\alpha$ -amino group of hydrophobic amino acids such as leucine, isoleucine and phenylalanine which bind in the  $S_1'$  specificity subsite [1-3]. Therefore the  $S_1'$  specificity site is thought to be the major determinant of substrate specificity in thermolysin with leucine giving optimal specificity in the  $S_1'$  subsite. The  $S_1$  specificity site also preferentially binds large hydrophobic residues such as phenylalanine [1-3]. It is therefore not surprising that Z-Phe is an inhibitor of thermolysin with  $K_i$  values of 0.5mM and 0.003 mM at pHs 7.2 and 5 respectively[4]. However, X-ray crystallographic studies have shown that with Z-Phe it is the carbobenzyloxyl protecting group (Z) and not the phenylalanyl phenyl group that is bound in the  $S_1'$  specificity pocket (Structure (b) in Scheme 1) and the alpha carboxylate group is directly coordinated to the active site zinc atom[5]. In contrast with Beta-Phenylpropionyl-L-phenylalanine(Beta-PPP) the Beta-propionyl phenyl ring of the N protecting group is not located in the  $S_1'$  specificity pocket. Instead it is the phenylalanyl sidechain which occupies the  $S_1'$  specificity pocket (Structure (a) in Scheme 1) and the alpha carboxylate group is not coordinated to the active site zinc atom[5]. Benzylsuccinic acid binds to thermolysin in a similar manner to beta-PPP[6].

Z-Trp is a potent inhibitor of the matrix metalloproteinase stromelysin[7]. X-ray crystallographic studies have shown that the tryptophan side chain can bind in the  $S_1$  specificity site of stromelysin (Structure (d) in Scheme 1) with the tryptophan alpha carboxylate group coordinated to the active site zinc atom[8]. However, X-ray crystallographic data suggests that while the  $S_1'$  subsite of thermolysin can accommodate the side chains of valine, leucine and phenylalanine it is too small to accommodate a tryptophan residue[9].

For thermolysin the phosphoramidate inhibitor P-Leu-Trp is  $\sim 100$  x more effective than P-Leu [10] showing that the binding of a tryptophan residue in the  $S_2'$  subsite (Structure (c) in Scheme 1) of thermolysin can improve inhibitor binding 100 fold. X-ray crystallographic analysis has shown that the tryptophan side chains of phosphoramidon [11] and the dipeptide inhibitor Val-Trp are both

bound in the S<sub>2</sub>' subsite of thermolysin[9]. Therefore we should consider the possibility that inhibitors such as Z-Trp could be bound in the S<sub>2</sub>' subsite of thermolysin so that their alpha carboxylate groups could not coordinate to the active site zinc atom.

In this study we show that Z-Trp is an effective inhibitor of thermolysin. We use NMR and spectrophotometric methods to determine whether the alpha carboxylate group of Z-Trp is coordinated to the active site zinc atom of thermolysin.

## **2. Materials and methods**

### *2.1. Materials.*

Two times crystallised thermolysin (Lot number T5KC991, 9340 proteinase units/mg as reported by the supplier) was supplied as a lyophilised powder by Daiwa Kasei, Japan. L-[1-<sup>13</sup>C]tryptophan (99 atom %) and L-[1-<sup>13</sup>C]phenylalanine (99 atom %) were obtained from Cambridge Isotope Laboratories, Inc. (50, Frontage Road, Andover, MA 01810-5413 USA). Phosphoramidon was obtained from Pepta Nova GmbH, Keplerstr. 26, 69207 Sandhausen, Germany. All other materials were obtained from Sigma-Aldrich Chemical Co., Gillingham, Dorset, U.K. The source of stromelysin-1 and the chemicals used to measure catalytic activity were as previously described [12].

### *2.2 Synthesis*

Z-[1-<sup>13</sup>C]-L-tryptophan and Z-[1-<sup>13</sup>C]-L-phenylalanine were synthesised by adding benzylchloroformate to an alkaline solution of the appropriate <sup>13</sup>C-enriched amino acid under Schotten-Bauman conditions [13].

### *2.3. Thermolysin samples and concentrations.*

In these experiments 130, 2 x101 and 51 mg of thermolysin were dissolved in 6 mL of 0.055 M Tris-HCl buffer at pH 7.4 containing 1.11 M NaSCN and 11.1 mM CaCl<sub>2</sub>. This solution was then

dialysed against 3 x 1 litre of the same buffer solution to remove the acetate from the NMR sample. On adding 10% (v/v)  $^2\text{H}_2\text{O}$  and adjusting the pH to 7.1 the resulting NMR sample had a large signal at 134 ppm due to NaSCN and signals at 61 and 62 ppm due to the Tris buffer. Additional broad signals at 180-170 ppm, 134-110 ppm and 70-10 ppm were due to the  $^{13}\text{C}$ -NMR signals from the carbonyl carbons, aromatic carbons and aliphatic carbons respectively of the 0.61 mM thermolysin at pH 7.0.

The concentration of protein was determined using an  $E_{280}$  of  $61000 \text{ M}^{-1} \text{ cm}^{-1}$  calculated using an  $E_{280}(1\%)$  of 17.65 [14] and an  $M_r$  value of 34600 [15].

#### 2.4. *Stromelysin-1 samples and concentrations*

The stromelysin-1 catalytic domain (residues 83-247) was prepared as described by Howe et al, [12]. Enzyme concentrations were determined using the  $\epsilon_{280}$  value of  $28700 \text{ M}^{-1} \text{ cm}^{-1}$  [12].

#### 2.5. *Preparation of Cobalt substituted Thermolysin and stromelysin-1*

Cobalt(II)-thermolysin was prepared using a procedure modified from that described by Holmquist [16, 17]. Crystals of native thermolysin (400.8 mg) were washed twice with 2-3 mL of distilled water by repeated suspension and centrifugation. The washed crystals at  $0^\circ\text{C}$  were then dissolved in 9 mL of 0.055 M Tris-HCl buffer at pH 7 containing 1.11 M NaSCN and 11.1 mM  $\text{CaCl}_2$ . This solution was dialysed for 24 hours against 3 x 225 mL of 2 mM 1,10-phenanthroline in 0.055 M Tris-HCl buffer at pH 7 containing 1.11 M NaSCN and 11.1 mM  $\text{CaCl}_2$  at  $4^\circ\text{C}$ . It was then dialysed against 450 mL of 0.2 mM 1,10-phenanthroline in 0.055 M Tris-HCl buffer at pH 7 containing 1.11 M NaSCN, 11.1 mM  $\text{CaCl}_2$  and 1mM  $\text{CoCl}_2$  at  $4^\circ\text{C}$ . The phenanthroline was then removed by dialysis against 450 mL of the same buffer, lacking the chelating agent. The excess of  $\text{CoCl}_2$  inside the dialysis tube was then removed by dialysis for 24 hours against 2 x 450 mL of the same buffer lacking  $\text{CoCl}_2$  (0.055 M Tris-HCl buffer at pH 7 containing 1.11 M NaSCN and 11.1

mM CaCl<sub>2</sub> at 4°C). It was then concentrated in a 10 mL Amicon ultrafiltration cell fitted with a Millipore PBGC Biomax ultrafiltration disc with a 10 kDa cutoff.

All buffers used were metal-free. The absence of metals was confirmed using a solution of 0.02% dithizone in CHCl<sub>3</sub>. This solution changes from a green colour to a purple colour if metals are present [18].

The activity of the cobalt substituted thermolysin was found to be 120-170% of that of native thermolysin which is similar to the activity of 80-280% reported previously for cobalt substituted thermolysin[17, 19, 20].

Cobalt substituted stromelysin-1 was prepared in the same way using active stromelysin-1 except that the dialysis buffer was changed to 20mM MOPS at pH 7.0. Cobalt substitution can increase or decrease catalytic activity from 36-217% depending on the substrate used[21]. The activity of the cobalt substituted stromelysin-1 towards Acetyl-Proline-Leucine-Glycine- [2-mercapto-4-methyl-pentanoyl]-Leucine-Glycine-OC<sub>2</sub>H<sub>5</sub> was found to be 32% of that of native stromelysin-1.

## *2.6. Catalytic activities of thermolysin and stromelysin-1*

The catalytic activity was measured using ~2 mM solutions of N-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide in 0.05 M Tris-HCl buffer at pH 7.5 containing 10 mM CaCl<sub>2</sub> and a thermolysin protein concentration of ~0.17 μM. The concentration of the substrate was determined using an E<sub>345</sub> of 766 M<sup>-1</sup>. cm<sup>-1</sup> [22] and its rate of hydrolysis was followed at 345 nm using a ΔE of 317 M<sup>-1</sup>.cm<sup>-1</sup> [22, 23]. Specific activities of stromelysin-1 with and without cobalt substitution were measured using Acetyl-Proline-Leucine-Glycine- [2-mercapto-4-methyl-pentanoyl]-Leucine-Glycine-OC<sub>2</sub>H<sub>5</sub> [12].

## *2.7. Determination of K<sub>i</sub> values at pH 7.0.*

0.1 mL of the inhibitor (*Z*-tryptophan) in dimethyl sulphoxide was added to 2.85 mL of 0.05 M Tris-HCl buffer at pH 7.0 containing 10 mM CaCl<sub>2</sub> and ~1.2 mM of the substrate (N-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide). 0.05 mL of ~12 μM thermolysin was added and the rate of decrease of absorbance at 345 nm was used to determine the rate of hydrolysis of the substrate. For stromelysin-1 K<sub>i</sub> values were determined as described previously[12] using the fluorescent substrate MCA-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys (Dnp)-NH<sub>2</sub>.

## 2.8. NMR samples

Thermolysin and stromelysin-1 samples all contained 10% of 99.9 atm% <sup>2</sup>H<sub>2</sub>O to obtain a deuterium lock signal. Thermolysin samples also contained 1.0 M NaSCN, 10 mM CaCl<sub>2</sub> and ~50mM Tris buffer. Stromelysin-1 samples contained ~9 mM CaCl<sub>2</sub> and the pH was maintained using ~25 mM MES, MOPS or acetate buffer at the appropriate pH.

All inhibitors were dissolved in d<sub>6</sub>-dimethyl sulfoxide and were added to enzyme samples to give final concentrations of 0.2-3.6% (v/v) d<sub>6</sub>-DMSO.

## 2.9. NMR spectra

NMR spectra at 11.75 T were recorded with a Bruker Avance DRX 500 standard-bore spectrometer operating at 125.7577 MHz for <sup>13</sup>C-nuclei.

The <sup>13</sup>C-NMR spectral conditions for the samples of *Z*-[1-<sup>13</sup>C]tryptophan in presence of 1 M NaSCN and thermolysin were: spectral width 221 ppm (32768 time-domain data points were used); acquisition time 0.59 s; relaxation delay 0.59 s; 90° pulse angle. Waltz-16 composite pulse <sup>1</sup>H decoupling with a BLARH100 amplifier was used with 25 dB attenuation during the acquisition time and 43 dB attenuation during the relation delay.

The <sup>13</sup>C-NMR spectral conditions for the samples of containing stromelysin-1 were: spectral width 237 ppm (32768 time-domain data points were used); acquisition time 0.55 s; relaxation delay 2.8 s;

90° pulse angle. Waltz-16 composite pulse  $^1\text{H}$  decoupling with a BLARH100 amplifier was used with 25 dB attenuation during the acquisition time and 43 dB attenuation during the relaxation delay. For samples of thermolysin or stromelysin-1 substituted with cobalt the spectral width was also increased up to 600 ppm so that we could determine whether our signals had undergone large paramagnetic shifts.

The  $^{13}\text{C}$  chemical shifts are quoted relative to tetramethylsilane at 0.00 p.p.m. The chemical shifts of  $d_6$ -dimethyl sulphoxide (< 5% (v/v)) at 38.7 ppm or of 1M NaSCN at 134 ppm were used as a secondary references in aqueous solutions.

### 3. Results

#### 3.1. Effect of 1M salts on the solubility of thermolysin.

The solubility of thermolysin in 50 mM Tris/HCl buffer containing 10 mM  $\text{CaCl}_2$  at pH 7.5 was ~1.1 mg/ mL which is good agreement with earlier reports which stated that the solubility was 1.0-1.2 mg/mL in 40 mM Tris/HCl buffer containing 10 mM  $\text{CaCl}_2$  at pH 7.5 [24]. High salt concentrations can be used to increase the solubility of thermolysin and sodium thiocyanate is one of the most effective salts at increasing the solubility of thermolysin e.g. 1M sodium thiocyanate increases the solubility of thermolysin 61 times to 73 mg/mL at 0°C though the solubility decreases to ~58 mg/mL at 37°C[24]. At 25°C we found that if ~150 mg of thermolysin was added per mL of 1 M NaSCN then after centrifuging there was 67 mg/mL of solution based on the absorbance at 280 nm which is a similar result to that obtained by Inouye [23] who prepared his solutions of thermolysin in a similar way.

High salt concentrations can increase both the solubility and catalytic activity of thermolysin[23-25]. For NaCl there is an exponential increase in the catalytic of thermolysin as the salt concentration is increased 11-17 fold up to 4 M NaCl [23-25]. However, sodium thiocyanate is a chaotropic salt which interacts strongly with peptide groups and so while it tends to solubilize

proteins by salting in the peptide group it can also denature proteins because it can interact more strongly with the unfolded denatured form of the protein rather than the folded native form of the protein[26]. Therefore it can promote denaturation at higher concentrations. This could explain why there is a three fold increase in the catalytic activity of thermolysin when the NaSCN concentration is increased to 1M, but there is a decrease in catalytic activity at higher concentrations of NaSCN[24]. Likewise aspartate transcarbamoylase is thought to be denatured by NaSCN concentrations greater than 1.25 M[27] . Therefore although NaSCN is highly effective at solubilising thermolysin care must be taken to ensure that denaturation does not occur. To ensure denaturation did not occur in our NMR studies on ligand binding we have used 1M NaSCN to solubilize thermolysin.

1.25M NaSCN was also found to decrease the affinity of aspartyl transcarbamoylase for N-(phosphonacetyl)-l-aspartate by ~800,000 fold while 1.25 M NaCl decreased it ~200,000 fold [27]. However, in our studies with thermolysin a 1M concentration of NaSCN only produced an ~2-fold increase in its  $K_i$  value from  $0.087 \pm 0.009$  mM to  $0.19 \pm 0.01$  mM for the inhibitor Z-tryptophan. Therefore it should be possible to use NMR to study ligand binding in the presence of high concentrations of NaSCN.

### *3.2. Effect of added salts on the signal-to noise of $^{13}\text{C}$ -NMR signals at 25°C in 10 mm NMR tubes*

A 10 mM solution of [1- $^{13}\text{C}$ ]glycine was prepared in 50 mM Tris/HCl buffer containing 10 mM  $\text{CaCl}_2$  at pH 7.5. The signal at 173.5 ppm from the  $^{13}\text{C}$ -enriched carboxylate carbon of glycine was used to determine how the addition of salts affected signal-to-noise at 25°C. Both sodium chloride and sodium thiocyanate had similar effects on signal-to-noise when they were added at concentrations of 0.5 to 2.5 M (Fig. 1). However, with sodium iodide under the same conditions the signal-to-noise was significantly lower (Fig. 1). Sodium thiocyanate is more effective than sodium chloride at increasing the solubility of thermolysin [24]. Therefore in subsequent NMR experiments 1 M NaSCN was used to solubilise thermolysin.

### *3.3. The binding of Z-tryptophan and Z-phenylalanine to thermolysin and cobalt substituted thermolysin at pH 7*

Z-tryptophan is an effective inhibitor of thermolysin with a  $K_i$  value of 0.087 mM at pH 7.0 and in 1M NaSCN this  $K_i$  value is approximately doubled (Table 1). Replacing the active site zinc atom with cobalt did not significantly change the  $K_i$  value for Z-tryptophan (Table 1). Therefore we estimate that in our NMR experiments using 0.59 mM thermolysin and 0.54 mM Cobalt substituted thermolysin with a 4-5 fold excess of Z-Trp more than 90% of the enzyme will be saturated with Z-tryptophan at pH 7. Z-tryptophan was tightly bound to thermolysin and cobalt substituted thermolysin at pH 5 (Table 1). Therefore it should be possible use NMR or spectrophotometric methods to directly observe Z-tryptophan bound to both thermolysin and cobalt substituted thermolysin at pHs 5 and 7. Z-phenylalanine is bound less tightly than Z-tryptophan at pHs 5 and 7 (Table 1). Cobalt substitution does not increase the  $K_i$  values for Z-phenylalanine at pHs 5 and 7 (Table 1). Therefore we should also be able to use NMR or spectrophotometric methods to directly observe Z-phenylalanine bound to thermolysin and cobalt substituted thermolysin at both pHs 5 and 7.

### *3.4. The binding of Z-tryptophan and Z-phenylalanine to stromelysin-1*

At pH 6.0 stromelysin-1 has maximal catalytic activity and Z-tryptophan is a good inhibitor of full length stromelysin1-477 with a  $K_i$  value of 6.3  $\mu$ M. Inhibition of the catalytic domains 100-273 and 100-264 by Z-tryptophan at pH 6.0 is ~3 times more and 4 times less effective respectively (Table1). At pHs 7.1 and 5.0 Z-tryptophan is still a good inhibitor of stromelysin100-264 with  $K_i$  values of 34  $\mu$ M and 52  $\mu$ M respectively (Table1). Substituting cobalt for zinc at pH 6 increased  $K_i$  by 50% and at pH 7 it increase  $K_i$  twofold (Table 1). Therefore we could use NMR and spectrophotometric methods to observe Z-tryptophan directly bound to stromelysin-1 or to cobalt substituted stromelysin-1.

### 3.5. $^{13}\text{C}$ -NMR of Z-[1- $^{13}\text{C}$ ]-L-tryptophan and Z-[1- $^{13}\text{C}$ ]-L-phenylalanine in the presence of thermolysin or stromelysin-1

On adding an excess (2.29 mM) of Z-[1- $^{13}\text{C}$ ]-L-tryptophan to thermolysin (Fig. 2a) at pH 7.0 a new signal at 182.7 ppm appeared (0.59 mM protein, Fig. 2b). This signal had a chemical shift 2.9 ppm larger than that of free Z-[1- $^{13}\text{C}$ ]-L-tryptophan at 179.8 ppm and was assigned to the signal from Z-[1- $^{13}\text{C}$ ]-L-tryptophan bound to the active site of thermolysin. To confirm this assignment we added phosphoramidon a good inhibitor of thermolysin ( $K_i$  28-60 nM, [10, 28-30]) which should displace the less potent inhibitor Z-[1- $^{13}\text{C}$ ]-L-tryptophan ( $K_i$  0.19 $\pm$  0.1 mM, Table 1) from the active site of thermolysin. Adding phosphoramidon (0.49 mM) led to a decrease in intensity of the signal at 182.7 ppm (Fig. 2c) confirming that this signal was due to Z-[1- $^{13}\text{C}$ ]-L-tryptophan bound to the active site of thermolysin.

A similar result was also obtained when Z-[1- $^{13}\text{C}$ ]-L-phenylalanine was incubated with thermolysin (data not shown) with the carboxylate carbon of the bound Z-[1- $^{13}\text{C}$ ]-L-phenylalanine having a chemical shift of 182.9 ppm at pH 7.0.

A similar result was also obtained when Z-[1- $^{13}\text{C}$ ]-L-tryptophan was incubated with stromelysin-1 (data not shown) with the carboxylate carbon of the bound Z-[1- $^{13}\text{C}$ ]-L-Tryptophan having a chemical shift of 182.1 ppm at pH 6.9.

### 3.6. Effect of pH on the NMR signals from Z-[1- $^{13}\text{C}$ ]-L-Phenylalanine bound to thermolysin and Z-[1- $^{13}\text{C}$ ]-L-tryptophan bound to thermolysin and stromelysin-1

The chemical shift of the  $^{13}\text{C}$ -enriched carboxylate carbon of Z-[1- $^{13}\text{C}$ ]-L-tryptophan in 1M NaSCN increased from 176.41 to 179.86 ppm as the carboxylate group ionised with a  $\text{pK}_a$  of 3.61 (Open circles in Fig. 3a). Excess free Z-[1- $^{13}\text{C}$ ]-L-tryptophan in the presence of thermolysin or stromelysin-1 titrated in the same way (Small filled circles in Fig. 3a). However, the signals due to the Z-[1- $^{13}\text{C}$ ]-L-tryptophan bound to thermolysin (182.82  $\pm$  0.04 ppm, small filled squares in Fig. 3b) and stromelysin-1 (182.87  $\pm$  0.06 ppm, large open squares in Fig. 3b) did not decrease from pH 6.9 to

4.5 (Fig. 3b). This shows that on binding to thermolysin and stromelysin-1 the pK<sub>a</sub> of the carboxylate group of Z-[1-<sup>13</sup>C]-L-tryptophan is decreased by at least 1.5 pK<sub>a</sub> units to a value of < 2.1 when it is bound to thermolysin or stromelysin-1.

Likewise when Z-[1-<sup>13</sup>C]phenylalanine was bound to thermolysin the chemical shift of its <sup>13</sup>C-enriched carboxylate group (182.10 ± 0.04 ppm) was unchanged from pH 4.4 to 6.9. The chemical shift of the carboxylate carbon of free Z-[1-<sup>13</sup>C]phenylalanine titrated from 176.19 ± 0.02 ppm to 179.51 ± 0.01 ppm according to a pK<sub>a</sub> of 3.50 ± 0.02 ppm. Therefore on binding to thermolysin the chemical shift of the <sup>13</sup>C-enriched carboxylate carbon of Z-[1-<sup>13</sup>C]phenylalanine has increased by 2.59 ppm. We conclude that when Z-[1-<sup>13</sup>C]phenylalanine is bound to thermolysin the pK<sub>a</sub> of its carboxylate group is reduced at least 1.5 pK<sub>a</sub> units to a value of < 2.

### 3.7. <sup>13</sup>C-NMR of Z-[1-<sup>13</sup>C]-L-tryptophan and Z-[1-<sup>13</sup>C]-L-phenylalanine in the presence of Cobalt substituted thermolysin or stromelysin-1

When excess Z-[1-<sup>13</sup>C]-L-tryptophan was added to thermolysin separate signals were seen (Fig. 2b) for the bound (182.7 ppm) and free inhibitor (179.8 ppm). However, when Z-[1-<sup>13</sup>C]-L-tryptophan was added to cobalt substituted thermolysin at pH 7.1 no signal was detected at 182.7 ppm (Fig. 2d). It has been shown that in cobalt substituted carbonic anhydrase direct ligation of a carboxylate group to the active site cobalt increased the linewidth of the carboxylate group to ~1100 Hz and increases its chemical shift by ~31 ppm due to its paramagnetic interaction with the unpaired electrons of the active site cobalt [31, 32]. In 1M NaSCN Z-[1-<sup>13</sup>C]-L-tryptophan binds equally strongly to zinc or cobalt substituted thermolysin (Table 1). Therefore the failure to detect a signal at 182.7 ppm (Fig. 2d) due to carboxylate carbon of the bound Z-[1-<sup>13</sup>C]-L-tryptophan with cobalt substituted thermolysin suggests that the inhibitor carboxylate group is in close proximity to the active site cobalt atom. If the inhibitor carboxylate group was directly coordinated to the cobalt atom as in carbonic anhydrase it would be expected that its chemical shift would be increased by ~30 ppm to a value of ~212.7 ppm and its linewidth would be ~1100Hz. There was no signal detected at

182.7 ppm or up to 220 ppm when Z-[1-<sup>13</sup>C]-L-tryptophan bound to cobalt substituted thermolysin (Fig. 2d). Likewise no signal was detected if the spectral width was increased to 600 ppm. As the signal-to noise of the signal at 182.7 ppm (Fig1b) is ~10 the signal would not be detected if there were a ten fold increase in linewidth to 100 Hz. Therefore we can conclude that the linewidth of this signal must be increased to at least 100 Hz by paramagnetic broadening by the active site cobalt of cobalt substituted thermolysin. Likewise when Z-[1-<sup>13</sup>C]-L-phenylalanine was added to cobalt substituted thermolysin at pH 7.0 the signal at 182.9 ppm was not observed (Spectra not shown). Also the signal at 182.1 ppm which was observed on adding Z-[1-<sup>13</sup>C]-L-tryptophan to stromelysin-1 at pH 6.9 was not observed when the catalytic zinc of stromelysin-1 was replaced with cobalt(spectra not shown).

No signal at ~182 ppm or up to 438 ppm was observed when Z-phenylalanine or Z-tryptophan was incubated with cobalt substituted thermolysin (data not shown).

### *3.8. Spectrophotometric studies of Z-[1-<sup>13</sup>C]-L-tryptophan and Z-[1-<sup>13</sup>C]-L-phenylalanine in the presence of cobalt substituted thermolysin*

At neutral pHs cobalt substituted thermolysin has a spectrum with a maximum absorption at 555 nm and a broad shoulder at at ~500nm(Fig. 4a). On adding  $\beta$ -phenylpropionyl-L-phenylalanine the intensity of the absorption at 550 nm increases ~2 fold and the shoulder at 500nm sharpens [17]. We have observed similar increases in absorbtion at 510 and 555 nm when Z-tryptophan is added to cobalt substituted thermolysin at pH 7.0 (Figs. 4a,b). Similar increases were also observed when Z-phenylalanine was added to cobalt substituted thermolysin (Figs 4c,d). The  $K_i$  value for Z-tryptophan binding to cobalt thermolysin at pH 7.0 was  $0.21 \pm 0.01$  in 1M NaSCN (Table 1) and under our experimental conditions (Fig. 4b) we calculate that 93% of the thermolysin will be saturated with Z-tryptophan. Making the appropriate correction to the observed extinctions we calculate that  $E_{510} = 113 \text{ M}^{-1} \text{ cm}^{-1}$  and  $E_{555} = 124 \text{ M}^{-1} \text{ cm}^{-1}$  for the complex formed between Z-tryptophan and cobalt substituted thermolysin. The observed extinctions for Z-phenylalanine binding

to thermolysin at pH 7.1 were larger ( $E_{510} = 158 \text{ M}^{-1} \text{ cm}^{-1}$  and  $E_{550} = 163 \text{ M}^{-1} \text{ cm}^{-1}$ ) for the complex formed between Z-phenylalanine and cobalt substituted thermolysin (Fig. 4d). If we assume that with Z-phenylalanine 1M salt doubles the  $K_i$  value from 0.16 mM (Table 1) to 0.32 mM (as was observed with Z-tryptophan, Table 1) then under our experimental conditions 80% of the thermolysin will be saturated with Z-phenylalanine (Fig. 4d) and so the extinctions when Z-phenylalanine is bound will be significantly larger ( $E_{510} = 198 \text{ M}^{-1} \text{ cm}^{-1}$  and  $E_{550} = 204 \text{ M}^{-1} \text{ cm}^{-1}$ ) than when Z-tryptophan is bound.

Similar spectrophotometric changes have been observed when cobalt substituted thermolysin[17] and carboxypeptidase A [33] bind B-phenylpropionyl-L-phenylalanine and phenylacetate respectively.

#### 4. Discussion

The  $S_1$  specificity site of thermolysin preferentially binds amino acid residues with hydrophobic side chains such as phenylalanine [2, 3] and so it is not surprising that Z-Phe is an inhibitor of thermolysin[4]. X-ray crystallographic studies have shown that with Z-Phe it is the carbobenzyloxyl protecting group (Z) and not the phenylalanyl phenyl group that is bound in the  $S_1'$  specificity pocket (Structure (b) in Scheme 1) and that the alpha carboxylate group is directly coordinated to the active site zinc atom[5]. On binding Z-[1- $^{13}\text{C}$ ]-L-phenylalanine to thermolysin the chemical shift of the  $^{13}\text{C}$ -enriched carboxylate group increased 2.6 ppm from 179.5 to 182.2 ppm. This suggests that direct coordination of its  $^{13}\text{C}$ -enriched  $\alpha$ -carboxylate group to the active site zinc increases its chemical shift by 2.6 ppm.

Z-tryptophan is a potent inhibitor of the matrix metalloproteinase stromelysin[7] and X-ray crystallographic studies have shown that the tryptophan side chain can bind in the  $S_1$  specificity site of stromelysin with the tryptophan  $\alpha$ -carboxylate group coordinated to the active site zinc atom[8]. When Z-[1- $^{13}\text{C}$ ]-L-tryptophan bound to stromelysin-1 the chemical shift of its  $^{13}\text{C}$ -enriched carboxylate carbon increased by 3.01 ppm from 179.86 to 182.87 ppm (Fig. 3). These results suggest

that when an  $\alpha$ -carboxylate group binds to the active site zinc atom of the metalloproteases thermolysin and stromelysin-1 its chemical shift will increase by 2.6-3.0 ppm.

Therefore the fact that the chemical shift of the  $^{13}\text{C}$ -enriched  $\alpha$ -carboxylate carbon of Z-[1- $^{13}\text{C}$ ]-L-tryptophan increased 2.9 ppm from 179.8 to 182.7 on binding to thermolysin (Figs. 2,3) suggests that its  $\alpha$ -carboxylate group is bound to the active site zinc of thermolysin. However, X-ray crystallographic data suggests that while the  $S_1'$  subsite of thermolysin can accommodate the side chains of valine, leucine and phenylalanine it is too small to accommodate a tryptophan residue[9]. X-ray crystallographic analysis have shown that the the tryptophan side chains of phosphoramidon [11] and the dipeptide inhibitor Val-Trp are both bound in the  $S_2'$  subsite of thermolysin[9].

If the  $\alpha$ -carboxylate of Z-[1- $^{13}\text{C}$ ]-L-tryptophan is as our NMR results suggest directly coordinated to the active site zinc of thermolysin then either the solution structure differs from the X-ray crystallographic structure[9] and the tryptophan side chain can bind in the  $S_1$  subsite or the tryptophan side chain does not have to bind in the  $S_1$  subsite when the alpha carboxylate group of Z-[1- $^{13}\text{C}$ ]-L-tryptophan coordinates to the active site zinc atom of thermolysin. A third possibility is that the the tryptophan side chain is bound in the  $S_2'$  subsite and the  $\alpha$ -carboxylate group interacts with some other positively charged group which like zinc increases the chemical shift of the  $^{13}\text{C}$ -enriched  $\alpha$ -carboxylate carbon of Z-[1- $^{13}\text{C}$ ]-L-tryptophan by  $\sim 3$  ppm.

Cobalt substituted thermolysin has been shown to have a very similar structure to wild type thermolysin[34] and our samples and those of other workers have similar catalytic activities to the wild type enzyme[17, 19]. Therefore our  $^{13}\text{C}$ -NMR results with Z-[1- $^{13}\text{C}$ ]-L-tryptophan binding to the cobalt substituted enzyme (Fig. 2) strongly support our suggestion that the carboxylate group of Z-[1- $^{13}\text{C}$ ]-L-tryptophan is in close proximity to the active site zinc atom of wild type thermolysin.

The  $^{13}\text{C}$ -enriched carboxylate carbon of carboxymethylated carbonic anhydrase is directly coordinated (3.1 Å) to the active site zinc of carbonic anhydrase at pH  $\sim 8$  [35]. On replacing the zinc atom with cobalt the chemical shift and linewidth of the  $^{13}\text{C}$ -enriched carboxylate carbon increases from  $\sim 174$  ppm and 5Hz[36] to  $\sim 207$  ppm and  $\sim 1100$  Hz respectively[32]. In our studies the signal at 182.7 ppm was not observed when Z-[1- $^{13}\text{C}$ ]-L-tryptophan was bound to cobalt substituted

thermolysin and no new signal was observed up to 220 ppm (Fig. 2d). The signal-to noise of the signal at 182.7 ppm was  $\sim 10$ . Therefore this signal would not be observed if there was a 10 fold increase in linewidth from  $16.6 \pm 2.7$  Hz to 166.7 Hz. From the results of Morley et al., [32] we estimate the signal-to noise of the signal with a linewidth of  $\sim 1100$  Hz to be  $\sim 6$  Hz when  $\sim 3$  mM carbonic anhydrase is used. In our experiments in 1M salt signal-to-noise will be  $\sim 50\%$  less (Fig. 1) and as we use  $\sim 0.6$  mM thermolysin they will be reduced a further 5 times. Therefore the signal-to noise in our experiments with cobalt substituted thermolysin will be  $\sim 10$  times less than the value of  $\sim 6$  obtained for the carboxylate carbon signal of linewidth  $\sim 1100$  Hz in the experiments with cobalt substituted carbonic anhydrase. We conclude that we will not be able observe a carboxylate carbon in our experiments if it has a linewidth of 160-1100 Hz.

Using the Solomon-Bloembergen equation [37]

$$LW = C (4T_c + 3T_c/(1+W_L^2.T_c^2) + 13T_c/(1+W_s^2.T_c^2))/2\pi r^6$$

where  $T_c$  is the electronic correlation time of cobalt ( $10^{-11}$  s $^{-1}$ ),  $W_L$  is the Larmour frequency of carbon,  $W_s$  is the electronic Larmour frequency of cobalt and  $r$  is distance in Å between the cobalt atom and the carboxylate carbon atom.  $C$  is a collection of constants. A  $C$  value of 666 was found to give a linewidth of 1100 Hz when  $r = 3.1$  Å as was observed in carbonic anhydrase [35]. Using this value of  $C$  with thermolysin we estimate that a linewidth of  $\sim 167$  Hz would be observed when  $r$  is 4.24 Å. Therefore we can conclude that the carboxylate carbon of Z-[1- $^{13}$ C]-L-tryptophan is  $\leq 4.24$  Å from the active site cobalt atom of thermolysin.

X-ray crystallography ((PDB: 1CIZ) has shown that the distance from the tryptophan carboxylate carbon of a sulphonamide inhibitor to the active site zinc atom of stromelysin-1 is only 2.71 Å [8]. If this is also true in carbonic anhydrase then the value of  $C$  would be reduced to 582.2 and then using this value of  $C$  we estimate that the distance of carboxylate carbon of Z-[1- $^{13}$ C]-L-tryptophan will be  $\leq 3.71$  Å from the active site cobalt atom of thermolysin.

Using X-ray crystallography the dipeptide Val-Trp [9] and the inhibitor phosphoramidon [11] have both been shown to bind with tryptophan bound in the S<sub>2</sub>' subsite and the distance from the tryptophan carboxylate carbon to the active site zinc atom is 6.91 Å (PDB 3TMN) and 7.39 Å (PDB: 1TLP) in these complexes respectively. At a distance of 6.9 Å the paramagnetic linebroadening due to cobalt would be only 9 Hz and the signal originally at 182.7 ppm would be observed as its signal-to-noise would only be reduced ~50% from ~10 to ~5. As this signal was not observed we can conclude that Z-[1-<sup>13</sup>C]-L-tryptophan is not bound in the S<sub>2</sub>' subsite as is observed with Val-Trp [9] and phosphoramidon [11].

If the carboxylate carbon-cobalt distance is 2.71 Å in thermolysin as in stromelysin then the linebroadening at a distance of 6.91 Å would only be 4 Hz and so the signal originally at 182.7 ppm would be easily seen in our experiments with only a 30% reduction in signal-to-noise. This supports our conclusion that Z-[1-<sup>13</sup>C]-L-tryptophan is not bound in the S<sub>2</sub>' subsite of thermolysin.

The ligand-field stabilization energy is smaller in tetrahedral cobalt(II) complexes than in octahedral cobalt(II) complexes and the extinction coefficients for octahedral complexes are much smaller (<30 M<sup>-1</sup>cm<sup>-1</sup>) than for tetrahedral complexes [38]. Therefore the large extinction coefficients observed when Z-phenylalanine or Z-tryptophan are bound to cobalt substituted thermolysin at pH 7 confirms that these ligands form tetrahedral complexes with active thermolysin at pH 7.

From our spectrophotometric results we conclude that the active site cobalt of cobalt substituted thermolysin is tetrahedrally coordinated at pH 7 when either Z-tryptophan or Z-phenylalanine are bound. From our experiments using cobalt substituted thermolysin we conclude that neither the side chains of Z-tryptophan or Z-phenylalanine bind in the S<sub>2</sub>' subsite of thermolysin. Our NMR results show that the carboxylate carbon of Z-[1-<sup>13</sup>C]-L-tryptophan is in close proximity to the active site metal of thermolysin and stromelysin. Likewise the carboxylate carbon of Z-[1-<sup>13</sup>C]-L-phenylalanine is in close proximity to the active site metal of thermolysin. The fact that the carboxylate carbons of both Z-[1-<sup>13</sup>C]-L-tryptophan and Z-[1-<sup>13</sup>C]-L-phenylalanine have chemical shifts of ~183 ppm when bound to thermolysin and stromelysin-1 (only Z-[1-<sup>13</sup>C]-L-tryptophan

binding studied) shows that they are all bound in a similar way, most probably with their carboxylate group directly coordinated to the active site zinc atom of thermolysin and stromelysin.

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## Figure legends

**Fig. 1.** Effect of added salts on the signal-to-noise of  $^{13}\text{C}$ -NMR signals at  $25^\circ\text{C}$  in 10 mm NMR tubes. Acquisition parameters were as described in the Materials and methods section. Signal-to-noise values were measured manually using the formula  $s/n = 2.5a/b$  where  $a$ =height of the signal due to the  $^{13}\text{C}$ -enriched carboxylate carbon of  $[1-^{13}\text{C}]$ glycine and  $b$ =width of the noise at 160-170 ppm. Each S/N value is expressed as percentage of S/N value in absence of salt. The lines connecting the experimental points were obtained by interpolation. Sample conditions were : 3 mL samples containing 1M NaSCN or NaCl or NaI, 20 mM  $[1-^{13}\text{C}]$ Glycine, 10% (v/v)  $^2\text{H}_2\text{O}$ , 50mM Tris HCl pH 7.5 buffer, and 10mM  $\text{CaCl}_2$ .

**Fig. 2.**  $^{13}\text{C}$ -NMR spectra of thermolysin in the presence of Z- $[1-^{13}\text{C}]$ -L-tryptophan in 10 mm NMR tubes. Acquisition parameters were as described in the Materials and methods section. 32768 transients were recorded for each spectrum and an exponential weighting factor of 20 Hz was used for all spectra. Sample conditions were: (a) 0.61 mM thermolysin, 1M NaSCN, 10 mM  $\text{CaCl}_2$ , 0.05 M Tris Buffer, 10% (v/v)  $\text{D}_2\text{O}$ , pH 7.1, 3.0 mL, (b) 0.59 mM thermolysin, 2.29 mM Z- $[1-^{13}\text{C}]$ Trp, 0.97 M NaSCN, 9.7 mM  $\text{CaCl}_2$ , 0.049 M Tris Buffer, 2.3% (v/v)  $d_6$ -dimethyl sulphoxide, 9.7% (v/v)  $\text{D}_2\text{O}$ , pH 7.0, 3.1 mL, (c) 0.57 mM thermolysin, 2.21 mM Z- $[1-^{13}\text{C}]$ Trp, 0.49 mM Phosporamidon, 0.94 M NaSCN, 9.4 mM  $\text{CaCl}_2$ , 0.047 M Tris Buffer, 3.6% (v/v)  $d_6$ -dimethyl sulphoxide, 9.4% (v/v)  $\text{D}_2\text{O}$ , pH 7.0, 3.2 mL, (d) 0.54 mM cobalt substituted thermolysin, 3.25 mM Z- $[1-^{13}\text{C}]$ Trp, 0.97 M NaSCN, 9.7 mM  $\text{CaCl}_2$ , 0.048 M Tris Buffer, 2.9% (v/v)  $d_6$ -dimethyl sulphoxide, 9.7% (v/v)  $\text{D}_2\text{O}$ , pH 7.1, 2.4 mL. 10 mm-diameter sample tubes were used for all samples.

**Fig. 3.** pH titrations of the chemical shift values of the  $^{13}\text{C}$ -NMR signals from free Z- $[1-^{13}\text{C}]$ Tryptophan and from Z- $[1-^{13}\text{C}]$ Tryptophan bound to thermolysin and stromelysin-1. Acquisition parameters were as described in the Materials and methods section.

(a) The continuous line was calculated using eqn. (1) :

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

pH titration of the signal at 176.41-179.86 p.p.m. due to the  $^{13}\text{C}$ -enriched carbon of Z-[1- $^{13}\text{C}$ ]Tryptophan (Large open circles). The continuous line was calculated using the fitted parameters  $pK_a = 3.61 \pm 0.01$ ,  $S_1 = 176.41 \pm 0.01$  p.p.m. and  $S_2 = 179.86 \pm 0.01$  p.p.m. Sample conditions were: 1.69 mM Z-[1- $^{13}\text{C}$ ]Tryptophan, 1M NaSCN, 10 mM  $\text{CaCl}_2$ , 0.05 M Tris Buffer, 3.3% (v/v)  $\text{d}_6$ -dimethyl sulphoxide, 10% (v/v)  $\text{D}_2\text{O}$ , pH 7.1, 3.0 mL. The pH was lowered using small aliquots of either 0.9M or 0.1M HCl containing 10% (v/v)  $^2\text{H}_2\text{O}$ .

Signals from the  $^{13}\text{C}$ -enriched carbon of the free Z-[1- $^{13}\text{C}$ ]Tryptophan (solid small circles) in the presence of thermolysin titrated in the same way as Z-[1- $^{13}\text{C}$ ]Tryptophan in isolation. Sample conditions are given below.

**(b)** pH titration of the signal at  $182.82 \pm 0.04$  ppm due to Z-[1- $^{13}\text{C}$ ]Tryptophan bound to thermolysin (small solid squares). Sample conditions were: 0.65 mM thermolysin, 1.66 mM Z-[1- $^{13}\text{C}$ ]Tryptophan, 0.98 M NaSCN, 9.8 mM  $\text{CaCl}_2$ , 0.049 M Tris Buffer, 1.64% (v/v)  $\text{d}_6$ -dimethyl sulphoxide, 9.8% (v/v)  $\text{D}_2\text{O}$ , pH 6.92, 3.0 mL. 5.67  $\mu\text{L}$  of 0.9 M HCl and 6.5  $\mu\text{L}$  of 0.1M HCl containing 10% (v/v)  $^2\text{H}_2\text{O}$  were added to give a sample at pH 6.55. Samples with pH values of 6.00, 5.52, 5.06, 4.52 and 4.11 were obtained by adding 65, 35, 50, 105 and 220  $\mu\text{L}$  of 0.1M HCl containing 10% (v/v)  $^2\text{H}_2\text{O}$  respectively.

pH titration of the signal at  $182.87 \pm 0.06$  ppm due to Z-[1- $^{13}\text{C}$ ]Tryptophan bound to Stromelysin-1 (Large open squares). Sample conditions were: pH 6.09, 0.69 mM stromelysin-1, 0.81 mM Z-[1- $^{13}\text{C}$ ]Tryptophan, 8.9 mM  $\text{CaCl}_2$ , 25.1 mM MOPS, 73.9 mM MES, 1.7% (v/v)  $\text{d}_6$ -dimethyl sulphoxide, 9.1% (v/v)  $\text{D}_2\text{O}$ , pH 6.92, total volume 352  $\mu\text{L}$ . Samples at pHs 5.58, 5.05 and 4.56 were prepared by adding 45  $\mu\text{L}$  of 1M MES(OH 5.6) and 8  $\mu\text{L}$  of sodium acetate buffer(pH 4.54), 12  $\mu\text{L}$  of sodium acetate buffer(pH 4.54), 3  $\mu\text{L}$  sodium acetate buffer(pH 4.3) and 27  $\mu\text{L}$  sodium acetate buffer(pH 4.11) respectively. A second stromelysin sample at pH 7.00 contained 0.76 mM stromelysin-1, 0.86 mM Z-[1- $^{13}\text{C}$ ]Tryptophan, 8.8 mM  $\text{CaCl}_2$ , 24.0 mM MOPS, 1.8% (v/v)  $\text{d}_6$ -

dimethyl sulphoxide, 9.8% (v/v) D<sub>2</sub>O, total volume 326  $\mu$ L. Samples at pHs 6.53, 6.07, 5.47 and 5.09 were prepared by adding 7  $\mu$ L of 1M MES(OH 5.6), 17  $\mu$ L of 1M MES(pH 5.6), 23  $\mu$ L of 1M MES(pH 5.6) and 16  $\mu$ L of 1M glycine buffer (pH 2.1), 5  $\mu$ L 1M glycine buffer (pH 2.1).

**Fig. 4.** Electronic absorption spectra of cobalt substituted thermolysin in the absence and in the presence of either Z-[1-<sup>13</sup>C]Tryptophan or Z-[1-<sup>13</sup>C]Phenylalanine at pH 7.1.

(a) 0.56 mM cobalt substituted thermolysin, 1.0 M NaSCN, 10.0 mM CaCl<sub>2</sub>, 0.050 M Tris Buffer, 10.0% (v/v) D<sub>2</sub>O, pH 7.1, 2.3 mL.

(b) 0.54 mM cobalt substituted thermolysin, 3.25 mM Z-[1-<sup>13</sup>C]Trp, 0.96 M NaSCN, 9.7 mM CaCl<sub>2</sub>, 0.048 M Tris Buffer, 2.9% (v/v) d<sub>6</sub>-dimethyl sulphoxide, 9.9 % (v/v) D<sub>2</sub>O, pH 7.1, 2.4 mL.

(c) 0.63 mM cobalt substituted thermolysin, 1.0 M NaSCN, 10.0 mM CaCl<sub>2</sub>, 0.050 M Tris Buffer, 10.0% (v/v) D<sub>2</sub>O, pH 6.9, 2.5 mL.

(d) 0.63 mM cobalt substituted thermolysin, 1.76 mM Z-[1-<sup>13</sup>C]Phe, 0.99 M NaSCN, 10.0 mM CaCl<sub>2</sub>, 0.05 M Tris Buffer, 0.24 % (v/v) d<sub>6</sub>-dimethyl sulphoxide, 10.0 % (v/v) D<sub>2</sub>O, pH 6.9, 2.5 mL.

### Scheme legends

**Scheme 1:** Binding of N-protected amino acids to thermolysin

<b>Table 1:</b> Disassociation constants for inhibitors of thermolysin or stromelysin-1					
Enzyme	Inhibitor	NaSCN (M)	pH	$K_i$ (mM)	
Zn <sup>++</sup> thermolysin	Phosphoramidon	0	7.2	0.00003	[10]
Zn <sup>++</sup> thermolysin	P-Leu-Trp	0	7.2	0.000015	[10]
Zn <sup>++</sup> thermolysin	$\beta$ -phenylpropionyl -L-phenylalanine	0	6.8	1.6	[17]
Zn <sup>++</sup> thermolysin	Z-phenylalanine	0	7.2	0.5	[4]
Zn <sup>++</sup> thermolysin	Z-phenylalanine	0	7.0	$0.37 \pm 0.06$	Present work
Co <sup>++</sup> thermolysin	Z-phenylalanine	0	7.1	$0.16 \pm 0.02$	Present work
Zn <sup>++</sup> thermolysin	Z-phenylalanine	0	5.1	$0.012 \pm 0.002$	Present work
Co <sup>++</sup> thermolysin	Z-phenylalanine	0	5.0	$0.008 \pm 0.001$	Present work
Zn <sup>++</sup> stromelysin 1-477	Z-tryptophan	0	6.0	0.0063	[7]
Zn <sup>++</sup> stromelysin100-273	Z-tryptophan	0	6.0	0.0021	[7]
Zn <sup>++</sup> stromelysin100-264	Z-tryptophan	0	6.0	$0.021 \pm 0.006$	Present work
Co <sup>++</sup> stromelysin100-264	Z-tryptophan	0	6.0	$0.030 \pm 0.004$	Present work
Zn <sup>++</sup> thermolysin	Z-tryptophan	0	7.0	$0.087 \pm 0.009$	Present work
Zn <sup>++</sup> thermolysin	Z-tryptophan	1	7.0	$0.19 \pm 0.01$	Present work
Co <sup>++</sup> thermolysin	Z-tryptophan	1	7.0	$0.21 \pm 0.01$	Present work
Zn <sup>++</sup> thermolysin	Z-tryptophan	0	5.0	$0.008 \pm 0.001$	Present work
Co <sup>++</sup> thermolysin	Z-tryptophan	0	5.0	$0.007 \pm 0.001$	Present work
Zn <sup>++</sup> stromelysin100-264	Z-tryptophan	0	7.1	$0.034 \pm 0.009$	Present work
Co <sup>++</sup> stromelysin100-264	Z-tryptophan	0	7.0	$0.071 \pm 0.010$	Present work
Zn <sup>++</sup> stromelysin100-264	Z-tryptophan	0	5.0	$0.052 \pm 0.019$	Present work

Figure 1

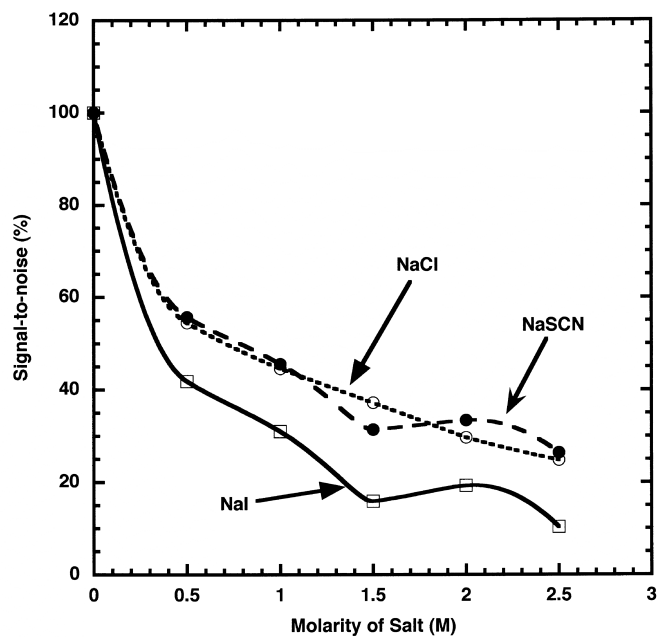


Figure 2

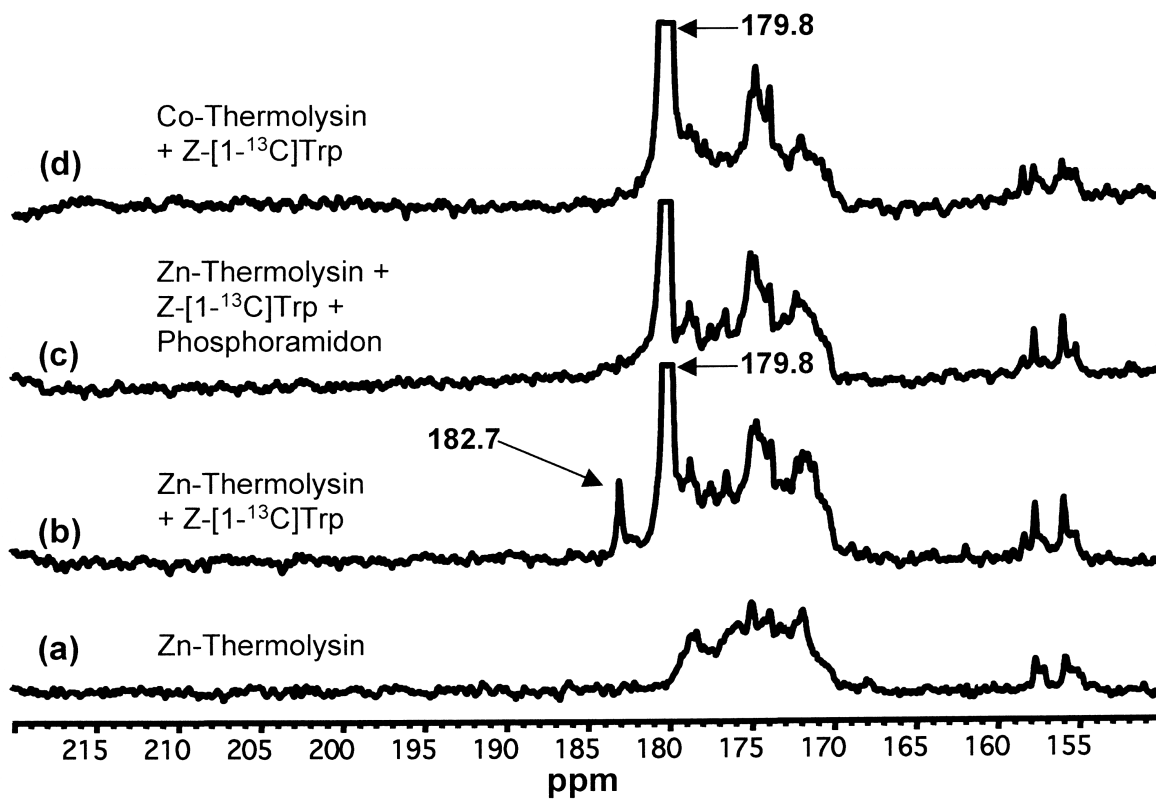


Figure 3

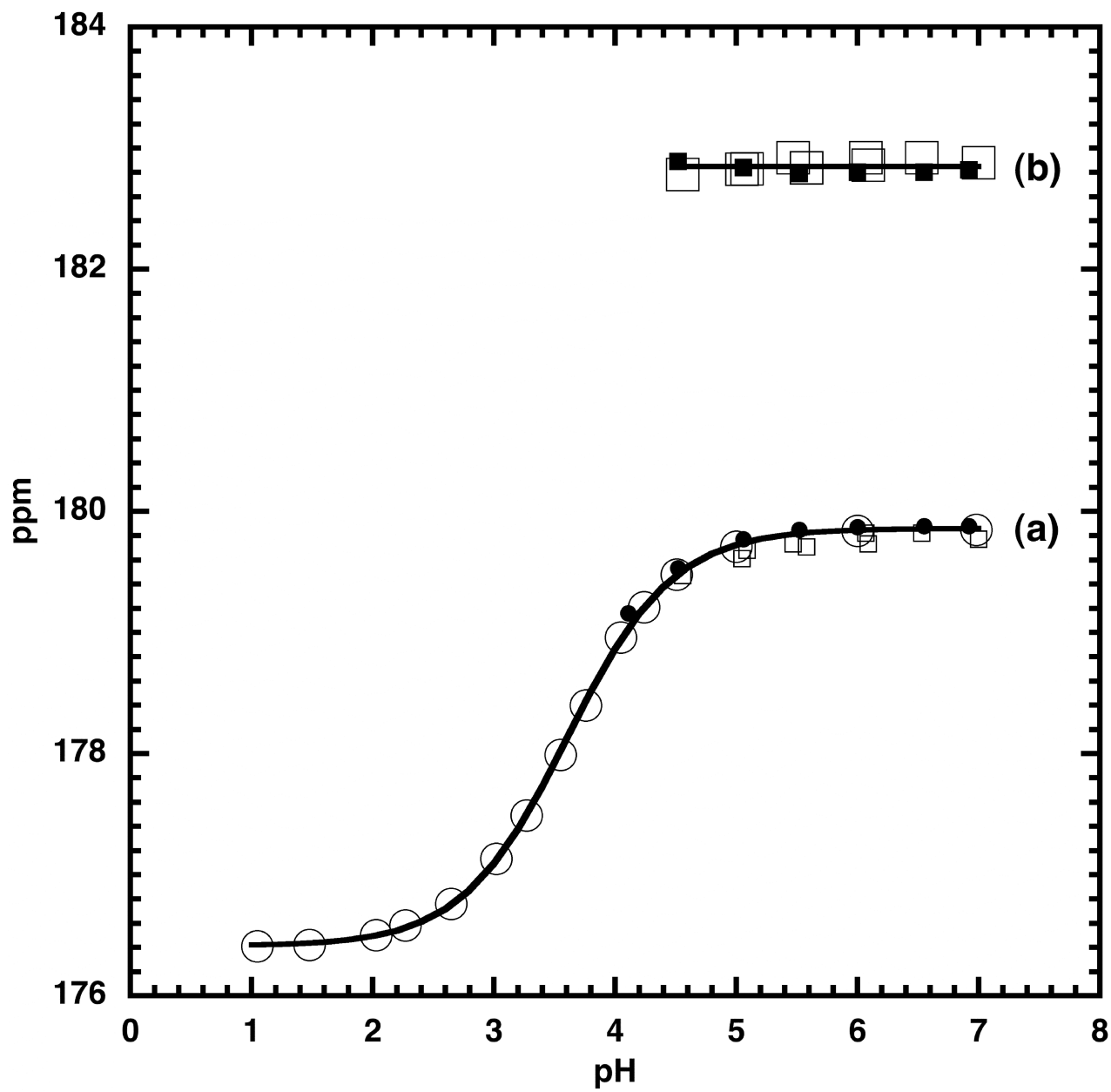
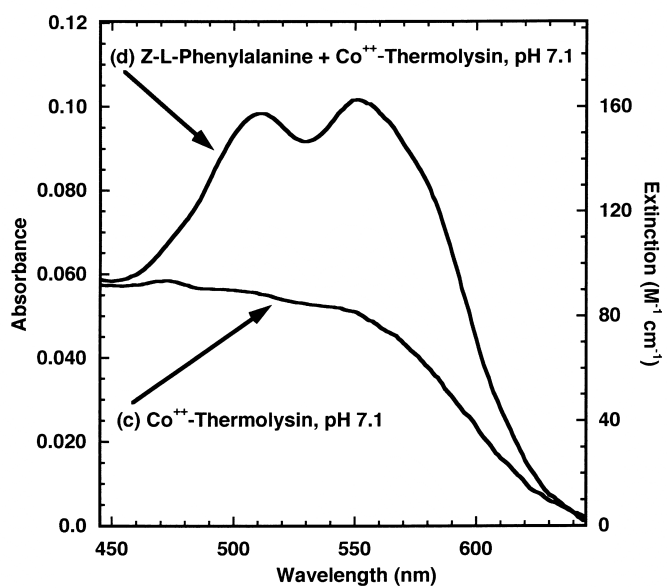
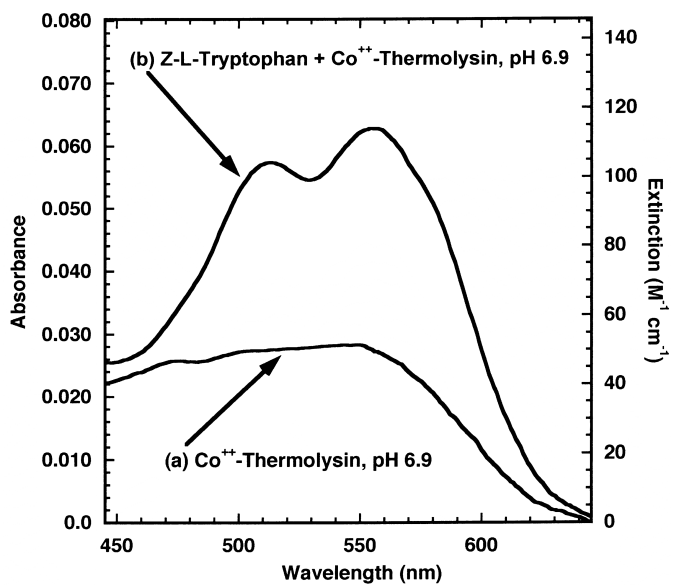
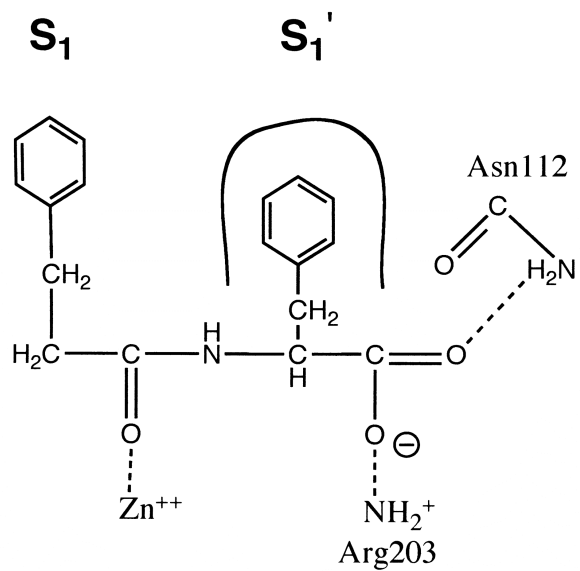


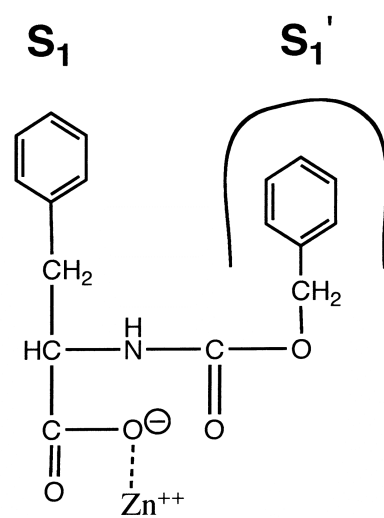
Figure 4



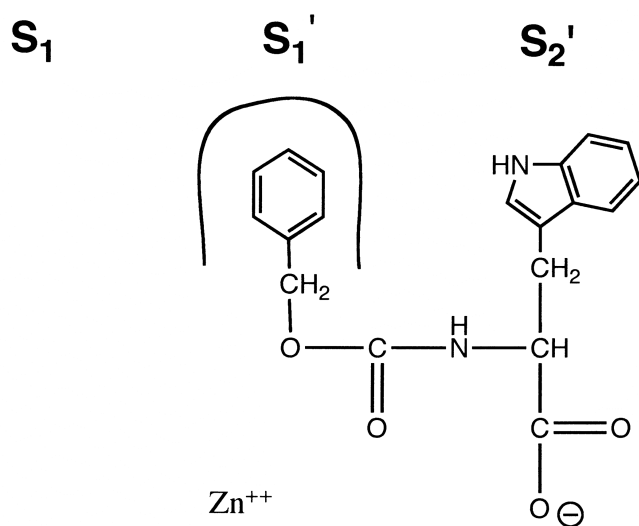
Scheme 1



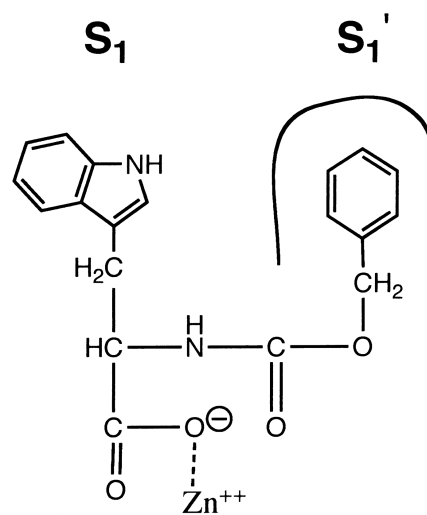
(a) Beta-phenylpropionyl-L-phenylalanine



(b) Z-Phenylalanine



(c) Z-Tryptophan



(d) Z-Tryptophan

## Highlights

The side chain of Z-Trp is not bound in the S<sub>2</sub>' subsite of thermolysin.

The pK<sub>a</sub> of carboxylate carbons are reduced by 1.5 pK<sub>a</sub> units on binding to thermolysin.

Z-Trp or Z-Phe induce tetrahedral coordination of the metal in thermolysin.

For both enzymes the inhibitor carboxylate groups are coordinated to the catalytic zinc.