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Structure/function relationships in the inhibition of thimet oligopeptidase by carboxyphenylpropyl-peptides

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Some novel *N*-[1(*RS*)-carboxy-3-phenylpropyl]tripeptide *p*-aminobenzoates have been synthesised as inhibitors of thimet oligopeptidase (EC 3.4.24.15). These compounds are considered to bind as substrate analogues with the Cpp group in S1 and the peptide portion in the S′ sites. The most potent inhibitor is Cpp-Ala-Pro-Phe-pAb, which has a K_i = 7 nM. Substitution of Gly for Ala at P1′ leads to weaker binding which can be ascribed to increased rotational freedom. Good substrates often have Pro at P2′ and Pro is favoured over Ala at this position in the inhibitors, too. When P2′ is Pro, Phe is preferred over Tyr and Trp in P3′. The *p*-aminobenzoate group makes an important contribution to the binding, probably by forming a salt bridge, and removal of the C-terminal negative charge results in much less potent inhibitors.

EC 3.4.24.15; Enzyme inhibition; Subsite specificity

1. INTRODUCTION

Thimet oligopeptidase (EC 3.4.24.15) is a thiol-dependent metallo-endopeptidase widely distributed in cells and tissues, and previously known by the names Pz-peptidase, endo-oligopeptidase A and soluble endopeptidase 24.15 [1]. Characteristic features of the enzyme are the inhibition by both chelators and thiol-reactive reagents and activation by low concentrations of thiols. The biological function of thimet oligopeptidase is unknown, but is capable in vitro of generating or destroying a number of pharmacologically active peptides and is found in the highest concentrations in the soluble protein fraction of testis, brain and pituitary [2]. Inhibitors may provide the best approach to investigating the physiological role of the enzyme [3].

Thimet oligopeptidase is selectively and reversibly inhibited by *N*-[1(*RS*)-carboxy-3-phenylpropyl]tripeptide *p*-aminobenzoates (Cpp-peptide-pAb) [4,5]. The mechanism of inhibition may be similar to that proposed for thermolysin and Cpp-Leu-Trp [6], where X-ray crystallography has revealed the coordination of the carboxyl of the Cpp group to the Zn atom in the active site of the enzyme. In thimet oligopeptidase, interaction of the 3-phenyl ring with the hydrophobic S1 subsite also makes an important contribution to the binding [4]. Cpp-peptide-pAb are competitive inhibitors, and since the most potent have peptide structures analogous to those of good substrates [4], the amino acid sidechains presumably occupy the same specificity subsites on the enzyme. By convention [7], these subsites are labelled S to the N-terminal side of the scissile bond and S′ to the C-terminal side, numbering being away from the bond cleaved, the scissile bond. The amino acid residues which occupy these subsites are labelled P and P′, respectively. Although it is not yet possible to define the characteristics of the subsites precisely [1], good substrates and inhibitors of thimet oligopeptidase commonly have hydrophobic residues at P1 and P3′ [2,4].

In addition to their use in biological experiments, inhibitors more potent than those currently available are needed for active site titration of the enzyme, so that kinetic studies can be placed on a quantitative basis. Also needed are inhibitors more resistant to degradation, which currently complicates their use in studies of the role of thimet oligopeptidase in vivo [3]. We now report our initial studies to investigate the relationships between inhibitor structure and function.

2. EXPERIMENTAL

2.1. *Materials*

Boc-amino acid *N*-hydroxysuccinimide esters, *p*-aminobenzoic acid and L-phenylalanine derivatives were from Sigma. Boc-amino acid *p*-aminobenzoates were made as described [8]. Ethyl 2-keto-4-phenylbutanoate from Scheizerhall (South Plainfield, NJ 07080) was saponified with NaOH to yield the sodium salt. Cpp-Ala-Ala-Tyr-pAb was a gift from Dr. M. Orlowski, Mount Sinai School of Medicine, New York, USA. Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) was made by solid phase peptide synthesis [9]. Rat testis thimet oligopeptidase was purified by a method similar to that used for the chicken enzyme [5], and gave a single band of 76 kDa in SDS/polyacrylamide gel electrophoresis.

Abbreviations: Ahx, L-2-aminohexanoic acid; Boc, *t*-butyloxycarbonyl; Cpp, *N*-[1((*RS*)-carboxy-3-phenylpropyl]; Dnp, dinitrophenyl; Mcc, 7-methoxycoumarin-3-carboxylyl; ONSu, *N*-hydroxysuccinimide ester; pAb, *p*-aminobenzoate; Pz, phenylazobenzyloxycarbonyl.

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2.2. *Synthesis of Cpp-Ala-Pro-Phe-pAb*

This was made step-wise by standard solution-phase methods [4,10]. Reactions were followed by HPLC on a C18 column, eluted with a gradient of 5–100% acetonitrile containing 0.1% trifluoroacetic acid (1ml/min) over 25 min. The column eluant was monitored at 230 nm. Boc-Phe-pAb (384 mg, 1 mmol) was treated with trifluoroacetic acid/water (19:1, v/v, 20 ml) for 20 min at 21°C to remove the Boc group. The acid was removed at 40°C in vacuo and the residue was dissolved in dimethylformamide (5ml). To this solution was added Boc-Pro-ONSu (330 mg, 1.1 mmol), 1-hydroxybenzotriazole (135 g, 1 mmol) and di-isopropylethylamine (0.7 ml, 4 mmol). After stirring overnight, analysis by HPLC showed the reaction to be complete. The dimethylformamide was evaporated at 40°C in vacuo and chloroform (50 ml) was added. After extraction with 20% (w/v) citric acid (2 × 20 ml), water (20 ml) and saturated NaCl (20 ml), the chloroform solution was dried ($MgSO_4$) and evaporated to leave crude Boc-Pro-Phe-pAb. This was recrystallised from ethanol/water (yielding 0.46 g, 95%) and treated with trifluoroacetic acid/water as described above. The residue after evaporation was dissolved in dimethylformamide and reacted with Boc-Ala-ONSu (286 mg, 1 mmol), 1-hydroxybenzotriazole and di-isopropylethylamine. As before, the solvent was evaporated after reaction overnight and the residue dissolved in chloroform, extracted and dried. The crude Boc-Ala-Pro-Phe-pAb was 97% pure by HPLC analysis and was treated with trifluoroacetic acid without further purification. The residue after evaporation was dissolved in methanol/water (1:1 v/v, 50 ml) and brought to pH 7 with 5 M NaOH. Sodium 3-phenyl-2-ketobutyrate (1 g, 5 mmol) and sodium cyanoborohydride (0.32 g, 5 mmol) were added and the mixture stirred at 21°C. HPLC analysis showed that reaction was complete after 24 h. The solvents were evaporated, water (50 ml) was added and the solution acidified with 6 M HCl (1.5 ml) to precipitate Cpp-Ala-Pro-Phe-pAb.HCl. The crude product was washed with ethyl acetate, and recrystallised from ethanol and water, yielding 0.33g (49%), m.p. 187–188°C. Analysis: calculated for $C_{43}H_{48}N_4O_8$.HCl (M_r 679.2) C, 62.72; H, 6.04; N, 8.60; found C, 62.98; H, 6.11; N, 8.60.

The other inhibitors were made by similar methods. The identities of the final peptide products were confirmed by elemental analysis, NMR or fast atom bombardment mass spectrometry. All the phenylalanine-containing inhibitors displayed characteristic double peaks on HPLC, attributable to the partially resolved *R* and *S* diastereomers [4].

2.3. *Determination of* K_i *values*

Thimet oligopeptidase activity was assayed at 30°C with the quenched fluorescent peptide Mcc-Pro-Leu+Gly-Pro-D-Lys(Dnp) [9], in which the symbol + represents the scissile bond. The fluorimeter was controlled, and data collected and analysed, by use of the FLUSYS software package [11]. The enzyme was pre-activated with 5 mM dithiothreitol in assay buffer (50 mM Tris-HCl, pH 7.8, containing 0.05% Brij 35) for 5 min at 21°C and then diluted 10-fold and kept at 0°C. Assays were made with 0.8 nM enzyme, based on protein concentration. For the determination of K_i, the rate of substrate hydrolysis in the absence of inhibitor was first recorded, and then the inhibitor was added in a negligible volume and the new steady state was monitored. Rapid equilibration was observed in all cases. Apparent inhibition constants $K_{i\,app}$ were calculated by fitting the fractional activity data to the Morrison equation [12] by nonlinear regression, using the program Enzfitter (Elsevier-Biosoft, Cambridge, UK). Values were corrected for the effect of substrate by using equation (1).

$$K_i = K_{i\,app}/(1 + [S]/K_m) \quad (1)$$

A value of 16 μM was determined for K_m. The differences in K_i values between inhibitors were converted into differences in the free energy of binding $66G_{bind}$ by equation (2).

$$66G_{bind} = -2.303RT \cdot \log(K_{i1}/K_{i2}) \quad (2)$$

Table 1

Effect of inhibitor structure at P1′

1	+ 1′	2′	3′	4′	K_i (nM)
Cpp	Phe	Ala	Phe	pAb	81 ± 10*
Cpp	Leu	Pro	Phe	pAb	279 ± 10
Cpp	Ala	Ala	Phe	pAb	30.6 ± 1.0
Cpp	Gly	Ala	Phe	pAb	774 ± 16
Cpp	Ala	Pro	Phe	pAb	7.3 ± 0.3
Cpp	Gly	Pro	Phe	pAb	289 ± 8
Cpp	Sar	Ala	Phe	pAb	15700 ± 200
Cpp	Sar	Pro	Phe	pAb	3410 ± 130

The Cpp-peptide-pAb inhibitors are substrate analogues and are thought to bind with the Cpp group in the S1 subsite of the enzyme. The residues have been numbered accordingly, and the symbol '+' indicates the bond corresponding to the scissile bond in a substrate. The error values are standard errors estimated by the Enzfitter program.

*Value from [3]

3. RESULTS AND DISCUSSION

3.1. *Alanine is favoured in P1′*

Good peptide substrates of thimet oligopeptidase commonly have hydrophobic residuesin P1 and P3′, and arginine is often found in P1′ [2], suggesting that the S1′ site of the enzyme is accessible to large side chains. This conclusion is supported by studies with inhibitors, since the K_i of Cpp-Phe-Ala-Phe-pAb is only 3-fold that of Cpp-Ala-Phe-pAb [4] (Table I). Leucine, however, is found to be an unfavourable residue in P1′.

It has been observed that replacement of Ala by Gly in P1′ to give Cpp-Gly-Ala-Phe-pAb results in a far less potential inhibitor [4,5], and we find similarly weak binding when P2′ is Pro (Table I). In principle, the weaker binding of the Cpp-Gly- inhibitors could be due to the lack of an interaction of the alanine side chain in the S1′ pocket of the enzyme, or to greater rotational freedom allowing the compounds to adopt unfavourable conformations. However, the K_i differences between Cpp-Ala-Ala-Phe-pAb and Cpp-Gly-Ala-Phe-pAb, and Cpp-Ala-Pro-Phe-pAb and Cpp-Gly-Pro-Phe-pAb correspond to differences in binding free energy $\Delta\Delta G_{bind}$ of 1.9 and 2.2 kcal/M, respectively. By contrast, the free energy change on transfer of a methyl group from octan-1-ol to water is only 0.7 kcal/M [13], which suggests that the hydrophobic pocket model is inadequate to explain the differences in K_i and that the increased flexibility of the glycine derivatives is responsible for their poorer binding. Such flexibility has been shown to be responsible for the weaker binding of analogues of Cpp-Ala-Pro (enalaprilat) to peptidyl-dipeptidase A (angiotensin converting enzyme) [14]. With these compounds, binding could be enhanced as much as 2500-fold by conformational restriction, so it seems reasonable to suggest that restricted rotation around the alanine N-Cα bond plays a role in orienting the Cpp carboxyl group towards the Zn atom in the active site

of thimet oligopeptidase, although other interactions probably contribute to efficient binding. For example, in the complex between thermolysin and Cpp-Leu-Trp (K_i = 50 nM [15]), both oxygens of the Cpp carboxyl group are coordinated to the zinc, but in addition each forms two hydrogen bonds to other residues lining the active site [6]. Similarly, the protonated secondary amino group forms three hydrogen bonds to surrounding residues [6]. The importance of this nitrogen in the interaction of Cpp-inhibitors with thimet oligopeptidase is shown clearly by the substitution of sarcosine (Sar) for Gly (Table I) which results in very marked losses of activity.

3.2. *Binding is favoured by proline in P2′*

Several good substrates by thimet oligopeptidase have Pro in P2′, including bradykinin and neurotensin [2], Bz-Gly-Phe+Ala-Pro-Phe-pAb [16], Pz-Pro-Leu+Gly-Pro-D-Arg [17] and the quenched fluorescent peptide, Dnp-Pro-Leu+Gly-Pro-Trp-D-Lys [18]. These data suggest that the binding of peptides to thimet oligopeptidase may be favoured not only by hydrophobic residues in P1 and P3′, but also by Pro in P2′. To investigate the effect of Pro in this position, we have compared some members of the series Cpp-Ala-Ala-X-pAb and Cpp-Ala-Pro-X-pAb, where X is an aromatic or large hydrophobic amino acid (Table II). Substitution of Pro for Ala leads to slightly tighter binding and Cpp-Ala-Pro-Phe-pAb is the most potent inhibitor of thimet oligopeptidase so far described with K_i = 7 nM.

Orlowski et al. [4] have observed that the binding of Cpp-Ala-Ala-Tyr-pAb to thimet oligopeptidase is twice as tight as that of the Phe analogue, but we do not see this effect when the P2′ residue is Pro and both Cpp-Ala-Pro-Tyr-pAb and Cpp-Ala-Ala-Tyr-pAb are comparable in inhibitory activity. By contrast, the inhibitor with Leu in P3′ shows a marked decrease in inhibitory potency (Table II).

3.3. *Potent inhibition requires a negatively charged C-terminus*

All the inhibitors discussed so far have been *p*-aminobenzoates. Stewart [19] has pointed out that the pAb group is structurally related to a dipeptide and this group seems likely to make a major contribution to the binding of the inhibitors. We explored the influence of

Table II

Effect of inhibitor structure at P2′ and P3′

1	+ 1′	2′	3′	4′	K_i (nM)
Cpp	Ala	Ala	Phe	pAb	30.6 ± 1.0
Cpp	Ala	Ala	Tyr	pAb	14.4 ± 0.5
Cpp	Ala	Pro	Phe	pAb	7.3 ± 0.3
Cpp	Ala	Pro	Tyr	pAb	11.8 ± 0.4
Cpp	Ala	Pro	Trp	pAb	17.6 ± 1.0
Cpp	Ala	Pro	Leu	pAb	76.8 ± 3.0

Table III

Effect of inhibitor structure at P4′

1	+ 1′	2′	3′	4′	K_i (nM)
Cpp	Ala	Ala	Phe	pAb	30.6 ± 1.0
Cpp	Ala	Ala	Phe	OH	155 ± 5
Cpp	Ala	Ala	Phe	NHPh	1860 ± 50
Cpp	Ala	Ala	Phe	$NHPhNO_2$	1000 ± 40
Cpp	Ala	Ala	Phe	NH_2	5790 ± 220

interactions at the C-terminus with a series of reagents in which the Cpp-Ala-Ala-Phe peptide part was retained (Table III). The most potent inhibitor remains the *p*-aminobenzoate, but surprisingly, removal of the pAb group to yield the free acid results only in a 5-fold increase in K_i, corresponding to the loss of 1.0 kcal/M of binding free energy. By contrast, replacement of the carboxyl group by a hydrogen atom to give the uncharged anilide results in binding weaker by 2.5 kcal/M. The *p*-nitroanilide inhibitor, with the dipolar nitro group in place of the carboxylate, is only marginally more effective than the anilide. The important contribution of the C-terminal negative charge to the binding is confirmed with the amide Cpp-Ala-Ala-Phe-NH_2, which binds more weakly than its parent acid by 2.2 kcal/M. These free energies are in the range of those observed for salt bridges in proteins [13] and suggest that, in addition to binding to the active site Zn, the *p*-aminobenzoate inhibitors have an important electrostatic interaction with lysine or arginine side chains in S4′ or S5′ subsites.

4. CONCLUSION

Although the studies described here have resulted in an inhibitor of thimet oligopeptidase that binds more tightly than those described before, Cpp-Ala-Pro-Phe-pAb binds too weakly to be used as an active site titrant, and a further decrease in K_i of about 20-fold will be necessary to achieve this aim. One approach to tighter inhibitors may be to explore alternatives to the Cpp group, perhaps by introducing substituents into the phenyl ring, as the 70-fold difference in inhibitory potency between carboxyphenylpropyl and carboxyphenylethyl derivatives of Ala-Ala-Phe-pAb [4] illustrates the dramatic effects of small changes in structure in this region of the molecule.

Cpp-Ala-Pro-Phe-pAb may prove to be a good inhibitor for in vivo studies of thimet oligopeptidase. Cpp-Ala-Ala-Phe-pAb is cleaved by neutral endopeptidase (EC 3.4.24.11) at the Ala-Phe bond [3], whereas the Pro-Phe bond may be more resistant to hydrolysis. This bond may be cleaved, however, by prolyl oligopeptidase (EC 3.4.21.26) and control experiments with specific inhibitors [20] of this enzyme will be necessary.

An advantage of the Cpp-peptide-pAb inhibitors is

their rapid binding to thimet oligopeptidase, so that equilibration is instantaneous on the 10 min time scale of our assays. By contrast, *N*-(phenylethylphosphonyl)-Gly-Pro-Ahx, designed as an inhibitor of *Clostridium histolyticum* collagenase [21], requires several minutes to reach equilibrium with thimet oligopeptidase, although the final K_i (7 nM), is similar (A.J. Barrett and M.A. Brown, unpublished observations). Likewise, phosphoramidon (*N*-[[(6-deoxy-α-L-mannopyranosyl)-oxy]hydroxyphosphinyl]-Leu-Trp) is a slow binding inhibitor of thermolysin [22], whereas Cpp-Leu-Trp binds normally [15].

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