Thimet oligopeptidase specificity: evidence of preferential cleavage near the C-terminus and product inhibition from kinetic analysis of peptide hydrolysis

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The substrate-size specificity of human thimet oligopeptidase (EC 3.4.24.15) was investigated with oligomers of glycyl-prolyl-leucine (GPL)_n where $n = 2, 3, 4$ and 5 . These peptides were cleaved only at Leu-Gly bonds to give GPL as the single final product. Hydrolysis was most rapid with (GPL)₃ and slowest with (GPL)₅. The more water-soluble oligomers of Gly-Hyp-Leu showed the same trend. (Gly-Hyp-Leu)₆ was not hydrolysed, consistent with the previous finding that substrates larger than 17 amino acids are not cleaved by thimet oligopeptidase. The cleavage of (GPL)₃ to GPL fitted a sequential first-order model. First-order kinetics were unexpected as the initial substrate concentration was greater than K_m . The anomaly was also seen during the cleavage of bradykinin and neurotensin, and in these

cases first-order behaviour was due to potent competitive inhibition by the C-terminal product. The sequential mechanism for (GPL)₃ breakdown by thimet oligopeptidase does not discriminate between initial cleavages towards the N- or C-terminus. As isoleucine is an unfavourable residue in P1, substrates were made in which selected leucine residues were replaced by isoleucine. GPL--GPI--GPL (where -- represents the bond between the tripeptide units) was resistant to hydrolysis and GPI--GPL--GPL was cleaved only at the -Leu-Gly- bond. Experiments with isoleucine-containing analogues of (Gly-Hyp-Leu)₄ showed that thimet oligopeptidase preferred to cleave these peptides near the C-terminus.

INTRODUCTION

A distinct subgroup of the endopeptidases show substrate-size restriction of their activities such that they hydrolyse oligopeptides or polypeptides but not proteins. These enzymes have been termed oligopeptidases [1]. Examples of mammalian oligopeptidases are thimet oligopeptidase (EC 3.4.24.15), prolyl oligopeptidase (EC 3.4.21.26), neprilysin (EC 3.4.24.11) and insulysin (EC 3.4.24.56). The substrates of mammalian oligopeptidases include many biologically active peptides, and there have been numerous reports presenting evidence that they mediate the catabolism of such peptides *in vivo* (reviewed for thimet oligopeptidase in [2]).

The mechanism of the substrate-size restriction of the activities of the oligopeptidases is poorly understood, although it presumably depends on the three-dimensional structures of the active sites. Morales and Woessner [3] provided evidence that collagen-derived peptides up to 30 residues are degraded by thimet oligopeptidase (then termed Pz-peptidase). In contrast, Dando et al. [4] were unable to detect cleavage of biologically active peptides larger than 17 residues. It is likely that the susceptibility to hydrolysis of a potential substrate molecule is determined by its shape and size in solution, not directly by the number of amino acid residues it contains, so it was not impossible that collagen-like peptides (containing repeats of the -Gly-Pro-Leu- motif) might show a different limit. Accordingly, we have now examined the action of thimet oligopeptidase on a series of collagen-like peptides that are oligomers of -Gly-Pro-Leu- and -Gly-Hyp-Leu-.

The kinetic analysis of the hydrolysis of several peptides revealed anomalous progress curves, the form of which is explained by significant inhibition of thimet oligopeptidase by the C-terminal products.

Dando et al. [4] noted that, although a C-terminus is not allowed in the P1' or P2' positions of a substrate, there is evidence for a favourable influence of a C-terminus in P3', P4' or P5'. In the present study we have been able to examine these influences in greater detail.

MATERIALS AND METHODS

Materials

Fluoren-9-ylmethoxycarbonyl (Fmoc)-amino acids and benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were from Calbiochem Novabiochem (U.K.) Ltd., Nottingham, U.K. 7-Methoxycoumarin-3-carboxyl (Mcc)-Pro-Leu-Gly-Pro-D-Lys(Dnp) [5], Fmoc-Lys(Dnp) [6] and 1-hydroxy-7-azabenzotriazole [7] were made as described. Fmoc-amino acid-poly(ethylene glycol) (PEG)-polystyrene (PS) and Fmoc-peptide amide linker (PAL)-PEG-PS resins were from Millipore, Watford, Herts., U.K. Human thimet oligopeptidase, homogeneous on SDS/PAGE, had a specific activity of 3.2 units/mg [4]. Bradykinin and neurotensin were from Sigma.

Collagen-like peptides

These were synthesized by the Fmoc method [8] on a Millipore 9050 Plus PepSynthesiser. Briefly, Fmoc-amino acids (0.4 mmol) were coupled to Fmoc-Leu-PEG-PS resin (0.1 mmol) by use of PyBOP (0.4 mmol), di-isopropylethylamine (0.8 mmol) and 1-hydroxy-7-azabenzotriazole (0.4 mmol) using a 20 min coupling cycle. Peptides were released from the resin by treatment with trifluoroacetic acid/water [19:1 (v/v), 20 ml] for 2 h at room temperature, applied to a reversed-phase HPLC column (22.5 mm × 250 mm) of Techogel 110-300 C18 (HPLC Tech-

Abbreviations used: Dnp, 2,4-dinitrophenyl; Fmoc, fluoren-9-ylmethoxycarbonyl; Mca, (7-methoxycoumarin-4-yl)acetyl; Mcc, 7-methoxycoumarin-3-carboxyl; P*, hydroxyprolyl; PAL, peptide amide linker; PEG, poly(ethylene glycol); PS, polystyrene; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; QF34, Mca-Gly-Gly-Phe-Leu-Arg-Arg-Ala-Lys(Dnp)-NH₂; QF37, Mca-Gly-Gly-Phe-Ile-Arg-Arg-Ala-Lys(Dnp)-NH₂; VIP, vasoactive intestinal peptide.

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nology) and eluted with a gradient of 5–65% acetonitrile in 0.1% trifluoroacetic acid (10 ml/min over 1 h). Fractions (5 ml) were analysed by HPLC (see below) and those containing homogeneous product were combined and freeze-dried from 10% (v/v) acetic acid.

Quenched fluorescent peptide substrates

QF 34 [(7-methoxycoumarin-4-yl)acetyl (Mca)-Gly-Gly-Phe-Leu-Arg-Arg-Ala-Lys(Dnp)-NH₂] and QF37 [Mca-Gly-Gly-Phe-Ile-Arg-Arg-Ala-Lys(Dnp)-NH₂] were synthesized similarly on Fmoc-PAL-PEG-PS resin. After deprotection of the second glycine, the resin (0.1 mmol) was suspended in dimethylformamide (3 ml) and treated with 7-methoxycoumarin-4-acetic acid, PyBOP and 1-hydroxy-7-azabenzotriazole (0.4 mmol of each) and di-isopropylethylamine (0.8 mmol). After reaction overnight, the insoluble excess of active ester was destroyed by the addition of piperidine (0.2 ml) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (0.2 ml). The peptides were released by treatment with trifluoroacetic acid/water/triethylsilane [18:1:1 (by vol.), 20 ml] and purified by preparative HPLC as described above.

Fragments of biologically active peptides

These were synthesized similarly by using the appropriate Fmoc-amino acid-PEG-PS resin. Peptide composition was confirmed by amino acid analysis by Dr. L. C. Packman, Department of Biochemistry, University of Cambridge, Cambridge, U.K.

HPLC assays of peptide hydrolysis

The rates of hydrolysis of peptides by human thimet oligopeptidase were determined at 37 °C with digestion mixtures containing 100 μM substrate in 50 mM Tris/HCl, pH 7.8, containing 0.01% Brij 35 and 0.1 mM dithiothreitol. The amount of enzyme was such that 70–90% cleavage occurred over about 2 h, as detected by HPLC. Samples (50 μl) were removed at timed intervals and treated with acetic acid (5 μl) to stop the digestion, before HPLC on a Perkin-Elmer Integral 4000 instrument equipped with a Techopak 10C18 column (4.6 mm × 250 mm) (HPLC Technology). A linear gradient of 5–100% acetonitrile containing 0.1% trifluoroacetic acid was run over 12.5 min at a flow rate of 2 ml/min and the eluate was monitored at 220 nm. For each time point, the areas of the peaks representing products and unchanged substrate were recorded. The concentration of substrate remaining [S] was calculated as

$$[S] = [S_0] \left(\frac{\text{Area of peak for unchanged substrate}}{\text{Total area of new peaks plus unchanged substrate}} \right)$$

where [S₀] is the initial substrate concentration (the sum of the peak areas remained constant during the course of the digestion). Similarly, the amounts of the intermediate products in a complex digestion were calculated from their fractions of the total peak areas. When the peaks of interest were below the threshold level of the integration software, a magnified image of the elution profile was printed. All the component peaks were cut out and weighed, and their proportions calculated from their fraction of the total weight. Products were identified by co-chromatography with authentic standards and by amino acid analysis.

Kinetic analysis of single cleavages

All progress curve data were analysed by non-linear regression (Enzfitter, Biosoft, Cambridge CB2 1LR, U.K.), the data within each series being normalized to the same enzyme activity [5].

When degradation occurred by a single cleavage, the changes in substrate concentration with time were analysed first by assuming simple first-order kinetics, according to eqn. (1):

$$[S] = [S_0]e^{-k_1t} \quad (1)$$

If the plot showed systematic deviations, as would be expected at initial substrate concentrations much greater than K_m , the rate of disappearance of substrate was analysed by the integrated form of the Michaelis and Menten equation:

$$t = \{([S_0] - [S])/V\} + \{(K_m/V)\ln([S_0]/[S])\} \quad (2)$$

where V is the maximum velocity.

The more general form of this equation, which takes into account the effects of competitive product inhibition [9,10], was also used:

$$t = \{(1 - K_m/K_p)([S_0] - [S])/V\} + \{(K_m/V)(1 + [S_0]/K_p)\ln([S_0]/[S])\} \quad (3)$$

where K_p is the competitive inhibition constant of the products.

Enzyme assays and determination of kinetic parameters

Thimet oligopeptidase was assayed with the quenched fluorescent substrate Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) as described previously [4,5]. Values for K_i were determined as described by Knight and Barrett [11] and corrected for the effect of substrate on the basis of simple competition according to eqn. (4):

$$K_i = K_{i(\text{app.})}/(1 + [S]/K_m) \quad (4)$$

When the inhibition is due to a competing substrate, the value of K_i is equivalent to K_m for that substrate [9].

Kinetic analysis of complex cleavages

Cleavage of the higher oligomers ($n > 2$) by thimet oligopeptidase produced complex sets of HPLC profiles, as the products of the initial cleavage were subsequently degraded. However, only Leu-Gly bonds were cleaved (see the Results and discussion section). Thus the cleavage of (GPL)₃ produced GPL and (GPL)₂, and the dimer was then hydrolysed to GPL. The overall reaction was treated as the sum of consecutive first-order reactions, according to the scheme:

Rate equations describing the changes in concentration of each component were derived by analogy with the standard model of consecutive first-order reactions, as reviewed in [12]:

$$[(\text{GPL})_3] = [(\text{GPL})_3]_0 e^{-k_1t} \quad (5)$$

$$[(\text{GPL})_2] = \left\{ \frac{[(\text{GPL})_3]_0}{k_1 - k_2} \right\} (e^{-k_1t} - e^{-k_2t}) \quad (6)$$

$$[(\text{GPL})] = [(\text{GPL})_3]_0 \left[3 - e^{-k_1t} - \frac{2(k_2e^{-k_1t} - k_1e^{-k_2t})}{k_2 - k_1} \right] \quad (7)$$

Degradation of higher oligomers introduces a further level of kinetic complexity and such results were not analysed in detail.

RESULTS AND DISCUSSION

Substrate-size restriction

To determine the action of thimet oligopeptidase on defined collagen-like peptides, we synthesized a series of substrates of increasing size containing repeats of the tripeptide sequences -Gly-Pro-Leu- and -Gly-Hyp-Leu-. These peptides were cleaved

Table 1 Apparent first-order rate constants for the cleavage of Gly-Pro-Leu oligomers by thimet oligopeptidase

Each rate constant, measured as described in the Materials and methods section, is that of the initial cleavage of the intact peptide. The substrate concentration was 100 μM .

Peptide	$10^4 \times$ Apparent first-order rate constant (s^{-1})
(GPL) ₂	2.43
(GPL) ₃	4.17
(GPL) ₄	1.65
(GPL) ₅	1.38

Figure 1 Digestion of GP*L oligomers by thimet oligopeptidase

The rate of disappearance of substrate was followed by HPLC, as described in the Materials and methods section. The data were fitted to eqn. (2), the integrated Michaelis-Menten equation, by non-linear regression. In each experiment, the initial concentration of substrate was 100 μM . ■, (GP*L)₂; ▲, (GP*L)₃; ▼, (GP*L)₄; ◆, (GP*L)₅.

exclusively at the -Leu-Gly- bond, which was not unexpected, as good substrates of thimet oligopeptidase, such as Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) [5], often have hydrophobic residues in positions P1 and P3' [13] and proline in P2' [4].

(GPL)₂, (GPL)₃, (GPL)₄ and (GPL)₅ were all cleaved to GPL, according to first-order kinetics. (GPL)₃ was cleaved most rapidly, but thereafter the rate decreased as the substrate size increased (Table 1). The insolubility of (GPL)₆ in water prevented its study as a substrate and a more soluble series of peptides was made by substituting hydroxyproline (P*) for proline. In this series the progress curves fitted eqn. (2), the integrated form of the Michaelis-Menten equation, more closely. As before, the trimeric nonapeptide (GP*L)₃ was cleaved most rapidly and the higher oligomers more slowly (Figure 1). No cleavage of (GP*L)₆ was seen, even during prolonged incubations with higher enzyme concentrations, although this peptide contains five potential cleavage sites. This behaviour is reminiscent of that seen previously with vasoactive intestinal peptide (VIP)-(10-28), which is not cleaved by thimet oligopeptidase, although VIP-(16-28) has two cleavage sites [4]. The present results confirm the conclusion drawn from that work that peptides of 18 or more residues cannot be cleaved by thimet oligopeptidase. This would suggest that if thimet oligopeptidase is involved in collagen degradation, it can only be after extensive degradation by other peptidases.

Figure 2 Time-dependence of the concentration of (GPL)₂ during the digestion of (GPL)₃ to GPL

The concentration of the intermediate (GPL)₂ was measured by HPLC. The overall reaction was treated as the sum of consecutive first-order reactions and the data were fitted to eqn. (6) by non-linear regression. The individual apparent first-order rate constants are given in Table 2.

Table 2 Apparent first-order rate constants calculated from (GPL)₃ progress curves

The digestion of (GPL)₃ by thimet oligopeptidase was assumed to be a sequential reaction, characterized by apparent first-order rate constants k_1 and k_2 , according to the scheme:

The changes in molar concentration of (GPL)₃, (GPL)₂ and GPL were fitted by non-linear regression analysis to eqns. (5), (6) and (7) respectively to give estimates for the rate constants.

Species observed	$10^4 \times$ Apparent first-order rate constants (s^{-1})	
	k_1	k_2
(GPL) ₃	4.17	—
(GPL) ₂	4.55	3.38
(GPL)	5.00	2.57

Sequential cleavage of (GPL)₃

When the cleavage of 100 μM (GPL)₃ to GPL was followed by HPLC, the amount of the intermediate (GPL)₂ was seen to pass through a maximum value (Figure 2), as expected for a sequential reaction [12]. Multiple peptide cleavages are often seen with thimet oligopeptidase [4], but the kinetics have not been studied in detail. Before kinetic analysis it was necessary to convert the fractional peak areas of the HPLC traces into molar concentrations. If each molecule of (GPL)₃ gave one molecule of GPL and one of (GPL)₂ (without further cleavage), the areas of the monomer and dimer peaks would be in a 1:2 ratio. However, their molar concentrations would be equal, and equal to the initial molar concentration of (GPL)₃ (100 μM). Thus $[(\text{GPL})_2] = 1.5 \times \text{fractional peak area} \times [S_0]$. Similarly, $[\text{GPL}] = 3 \times \text{fractional peak area} \times [S_0]$.

The progress curves for (GPL)₃, (GPL)₂ and GPL were fitted to eqns. (5), (6) and (7) respectively by non-linear regression to give values of the rate constants k_1 and k_2 (Table 2). The values

Table 3 K_m values of GPL oligomers, bradykinin and neurotensin

K_m values were determined by competitive inhibition of the hydrolysis of Mcc-Pro-Leu-Gly-Pro-Lys(Dnp) as described in the Materials and methods section.

Substrate	K_m (μM)
GPL	73.2
(GPL) ₂	48.0
(GPL) ₃	14.9
(GPL) ₄	13.5
(GPL) ₅	15.4
Bradykinin	0.38
Neurotensin	1.64

Figure 3 First-order degradation of bradykinin by thimet oligopeptidase

The concentration of bradykinin at each time point was measured by HPLC. The data were fitted to eqn. (1) by non-linear regression.

were in excellent agreement and validate the first-order sequential model of the degradation of (GPL)₃ by thimet oligopeptidase.

Why are the progress curves first-order?

First-order progress curves, such as were obtained for the hydrolysis of 100 μM (GPL)₃ by 0.8 nM (0.2 m-units/ml) thimet oligopeptidase, are expected according to Michaelis–Menten kinetics only when $[S_0] \ll K_m$. However, when the GPL oligomers were added as competing substrates in thimet oligopeptidase assays with Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp), the K_m (as K_i) values obtained (Table 3) were less than the initial substrate concentrations in the digests.

This kinetic anomaly was even more marked in the digestion of bradykinin and neurotensin by thimet oligopeptidase. Their progress curves starting at 100 μM were clearly first-order, as is shown for bradykinin in Figure 3, and yet these peptides were effective competitive substrates in the quenched fluorescence assays, with K_m values in the region of 1 μM (Table 3).

The anomaly can be resolved by postulating that in these cases the products of the cleavage reaction are competitive inhibitors. Laidler [9] noted that when the products bind with comparable affinity to the substrate, so that $K_p \approx K_m$, eqn. (3) reduces to the simple first-order form:

$$t = \left(\frac{K_m + [S_0]}{V} \right) \ln([S_0]/[S]) \quad (8)$$

Figure 4 Product inhibition during the degradation of bradykinin by thimet oligopeptidase mimics first-order behaviour

The data shown in Figure 3 were fitted to eqn. (3), the general form of the integrated Michaelis–Menten equation, which takes into account the effects of competitive product inhibition.

Table 4 Inhibition of thimet oligopeptidase by the cleavage products of bradykinin and neurotensin

Thimet oligopeptidase cleaves bradykinin to give R-P-P-G-F and S-P-F-R. Neurotensin is cleaved to give < E-L-Y-E-D-K-P-R and R-P-Y-I-L. The K_i values of these fragments were determined as described in the Materials and methods section.

Peptide	K_i (μM)
R-P-P-G-F	16.5
S-P-F-R	1.2
< E-L-Y-E-D-K-P-R	> 1000
R-P-Y-I-L	3.5

Thus under these conditions, product inhibition will give first-order progress curves, with a rate constant that varies with the initial substrate concentration, according to eqn. (9):

$$k_{1(\text{app.})} = V/([S_0] + K_m) \quad (9)$$

As the products of the (GPL)₃ digestion bind to thimet oligopeptidase with comparable affinity with their precursors (Table 3), the sequential first-order model of the degradation can be explained.

When the first-order progress curve for the hydrolysis of 100 μM bradykinin by 0.8 nM thimet oligopeptidase was analysed according to eqn. (3), an equally good fit was obtained (Figure 4) and the following values of the kinetic constants were calculated: $V = 15.3 \text{ nM} \cdot \text{s}^{-1}$; $K_m = 1.71 \mu\text{M}$; $K_p = 1.08 \mu\text{M}$. Similarly, the progress curve for hydrolysis of 100 μM neurotensin by 0.8 nM thimet oligopeptidase gave the values $V = 12.5 \text{ nM} \cdot \text{s}^{-1}$; $K_m = 2.7 \mu\text{M}$; $K_p = 1.2 \mu\text{M}$. These K_m values are in reasonable agreement with those determined by competitive inhibition (Table 3). In order to measure the K_p values directly, we synthesized the products of the enzymic reaction. Thimet oligopeptidase cleaves bradykinin at the -Phe-Ser- bond to give the fragments R-P-P-G-F and S-P-F-R, and neurotensin is cleaved at the -Arg-Arg- bond to give < E-L-Y-E-D-K-P-R and

Table 5 Digestion of isoleucine-containing peptides by thimet oligopeptidase

Oligomers of GP*L were synthesized in which selected leucine residues were replaced by isoleucine. The Ile-Gly bond is resistant to hydrolysis by thimet oligopeptidase. Potential cleavage sites that remained unhydrolysed are represented by the symbol $\approx$. Sites of hydrolysis are shown as +.

Peptide	Final products
1. GP*L + GP*L	GP*L
2. GP*I-GP*L	No cleavage
3. GP*L + GP*L + GP*L	GP*L
4. GP*I-GP*I-GP*L	No cleavage
5. GP*I-GP*L + GP*L	GP*I-GP*L, GP*L
6. GP*L $\approx$ GP*I-GP*L	No cleavage
7. GP*L + GP*L + GP*L + GP*L	GP*L
8. GP*L + GP*L + GP*I-GP*L	GP*I-GP*L, GP*L
9. GP*I-GP*L + GP*I-GP*L	GP*I-GP*L
10. GP*L $\approx$ GP*I-GP*I-GP*L	No cleavage

R-P-Y-I-L. When these peptides were tested as inhibitors of thimet oligopeptidase in the quenched fluorescence assay, those corresponding to the C-terminal fragments were found to be inhibitors, with K_i values similar to the K_p values calculated from the progress curves (Table 4).

Initial sites of cleavage

When a peptide is hydrolysed by thimet oligopeptidase at a single site, this is often located three or four residues from the C-terminus [4]. Taken with the work of Knight and Barrett [11], who observed the favourable influence of a negative charge at the C-terminus of inhibitors of thimet oligopeptidase, this could suggest that the enzyme has a preference for a carboxyl group in P3' or P4'. When two or more bonds are broken, the picture is less clear, as the products seem to appear in parallel [4], suggesting that the initial cleavage is rate-limiting. There has been no firm evidence to suggest that this initial cleavage occurs preferentially near either the N- or C-terminus.

To investigate whether such selectivity occurs, we used oligomers of GP*L in which isoleucine was substituted for leucine in P1 at the scissile bond. Dando et al. [4] observed that thimet oligopeptidase cleaved the quenched fluorescent peptide 2-amino-benzoyl-Gly-Gly-Phe-Leu-Arg-Arg-Val-NH(CH₂)₂NHDnp [14] at the -Leu-Arg- bond. We prepared an analogous substrate, QF34 [Mca-Gly-Gly-Phe-Leu-Arg-Arg-Ala-Lys(Dnp)-NH₂], which was also cleaved at -Leu-Arg- by thimet oligopeptidase. As isoleucine is not seen at P1 in the peptide substrates surveyed by Dando et al. [4], we synthesized QF37 in which leucine was replaced by isoleucine, in the expectation of a much slower cleavage at the same position. The rate of cleavage was indeed slower, but occurred at the -Arg-Arg- bond and there was no evidence of cleavage at -Ile-Arg-.

The unfavourable nature of isoleucine in P1 was confirmed when GP*I--GP*L (peptide 4 in Table 5 where -- represents the bond between tripeptide units) was found to be resistant to hydrolysis by thimet oligopeptidase. When GP*I--GP*L--GP*L (peptide 5 in Table 5) was incubated with the enzyme, there was rapid cleavage to the stable products GP*I-GP*L and GP*L. In contrast, GP*L--GP*I--GP*L (peptide 6) remained undigested under the same conditions, despite the potential Leu-Gly cleavage site near the N-terminus. These results suggest that thimet oligopeptidase might cleave such oligomeric peptides preferentially at C-terminal sites.

This aspect of the specificity was explored further with isoleucine analogues of (Gly-Hyp-Leu)_n (Table 5). When GL*L--GP*L--GP*I--GP*L (peptide 8) was treated with thimet oligopeptidase, the final products were GP*I--GP*L (peptide 2) and GP*L, via the intermediate GP*L--GP*L (peptide 1). If cleavage was preferentially about three residues from the C-terminus, in these oligomers it must be exactly there. After all, Dando et al. [4] did not find any single cleavages further than five residues from the C-terminus. So one would predict that GP*L--GP*L--GP*I--GP*L (peptide 8) would be stable to hydrolysis by thimet oligopeptidase. In practice, the product pattern showed that the initial cleavage occurred at the central -Leu-Gly- bond, six residues from both the N- and the C-terminus. It should be noted that hydrolysis of the N-terminal -Leu-Gly- bond would lead to an accumulation of the stable GP*L--GP*I--GP*L (peptide 6) and this was not seen, so once again a potential N-terminal cleavage site in an oligomeric substrate remained uncleaved. The ability of thimet oligopeptidase to cleave a bond located six residues from both the N- and C-terminus was also demonstrated with GP*I--GP*L--GP*I--GP*L (peptide 9), which produced only GP*I--GP*L (peptide 2). The exclusion of a potential N-terminal site was observed again with GP*L--GP*I--GP*I--GP*L (peptide 10), which remained undigested. It was somewhat surprising that the -Leu-Gly- bond remained uncleaved in GP*L--GP*I--GP*L (peptide 6), as the hydrolysis of GP*L--GP*L--GP*I--GP*L (peptide 8) and GP*I--GP*L--GP*I--GP*L (peptide 9) showed that cleavage six residues from the C-terminus can occur. It may well be that there are additive negative influences from the proximity of the N-terminus and the presence of isoleucine in P3'.

The pattern of cleavage of natural and synthetic peptides by human thimet oligopeptidase has been discussed previously [4]. In all cases, even in peptides with multiple cleavage sites, the scissile bonds were located at least three residues from a free N-terminus. We have shown here that this condition is not sufficient in itself to ensure that cleavage takes place, but we suggest that the nature of the C-terminal sequence exerts a controlling influence. The site of the initial cleavage can be up to six residues from the C-terminus, which is one residue more than previously seen [4].

The present results suggest a model for the multiple cleavages seen in some peptides [4]. We would propose that the initial cleavage takes place towards the C-terminus of the intact peptide and is followed by a second more rapid cleavage at a position that depends on the nature of the new C-terminal sequence.

Thus thimet oligopeptidase could be regarded as a peptidyl-peptidase, releasing C-terminal fragments of up to six residues. From that point of view, it is interesting to note that another oligopeptidase, neprilysin, often behaves as a peptidyl-dipeptidase. Moreover, neprilysin is inhibited by dipeptides that can be C-terminal products [15,16].

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REFERENCES

- Barrett, A. J. and Rawlings, N. D. (1992) *Biol. Chem. Hoppe-Seyler* **373**, 353–360
- Barrett, A. J., Brown, M. A., Dando, P. M. et al. (1995) *Methods Enzymol.* **248**, in the press
- Morales, T. I. and Woessner, J. F., Jr. (1977) *J. Biol. Chem.* **252**, 4855–4860
- Dando, P. M., Brown, M. A. and Barrett, A. J. (1993) *Biochem. J.* **294**, 451–457
- Tislijar, U., Knight, C. G. and Barrett, A. J. (1990) *Anal. Biochem.* **186**, 112–115

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- 6 Anastasi, A., Knight, C. G. and Barrett, A. J. (1993) *Biochem. J.* **290**, 601–607
 - 7 Carpino, L. A. (1993) *J. Am. Chem. Soc.* **115**, 4397–4398
 - 8 Atherton, E. and Sheppard, R. C. (1989) *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, Oxford
 - 9 Laidler, K. J. (1958) *The Chemical Kinetics of Enzyme Action*, pp. 87–89 and 101–102, Oxford University Press, Oxford
 - 10 Cornish-Bowden, A. (1976) *Principles of Enzyme Kinetics*, pp. 143–146, Butterworths, London
 - 11 Knight, C. G. and Barrett, A. J. (1991) *FEBS Lett.* **294**, 183–186
 - 12 Cox, B. G. (1994) *Modern Liquid Phase Kinetics*, pp. 27–28, Oxford University Press, Oxford
 - 13 Chu, T. G. and Orlowski, M. (1985) *Endocrinology* **116**, 1418–1425
 - 14 Juliano, L., Chagas, J. R., Hirata, I. Y. et al. (1990) *Biochem. Biophys. Res. Commun.* **173**, 647–652
 - 15 Schwartz, J.-C., Costentin, J. and Lecomte, J.-M. (1985) *Trends Pharmacol. Sci.* **6**, 472–476
 - 16 Kim, Y.-A., Shriver, B., Quay, T. and Hersh, L. B. (1992) *J. Biol. Chem.* **267**, 12330–12335
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