Human thimet oligopeptidase

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We have purified human thimet oligopeptidase to homogeneity from erythrocytes, and compared it with the enzyme from rat testis and chicken liver. An antiserum raised against rat thimet oligopeptidase also recognized the human and chicken enzymes, suggesting that the structure of the enzyme has been strongly conserved in evolution. Consistent with this, the properties of the human enzyme were very similar to those for the other species. Thus human thimet oligopeptidase also is a thiol-dependent metallo-oligopeptidase with M_r about 75000. Specificity for cleavage of a number of peptides was indistinguishable from that of the rat enzyme, but K_i values for the four potent reversible inhibitors tested were lower. In discussing the results, we consider the determinants of the complex substrate specificity of thimet oligopeptidase. We question whether substrates containing more than 17 amino acid residues are cleaved, as has been suggested. We also point out that the favourable location of a proline residue and a free C-terminus in the substrate may be as important as the hydrophobic residues in the P2, P1 and P3′ positions that have been emphasized in the past.

INTRODUCTION

Thimet oligopeptidase (EC 3.4.24.15) is a thiol-dependent metallo-endopeptidase that has previously been known as Pz-peptidase, endo-oligopeptidase A and soluble metalloendopeptidase (Barrett and Tisljar, 1989; Tisljar et al., 1989; Barrett and Brown, 1990; Barrett, 1991). There is evidence that the enzyme plays a role in the processing or catabolism of a variety of important biologically active peptides, including enkephalin precursors (Chu and Orlowski, 1985; Cicilini et al., 1988), bradykinin (McDermott et al., 1987), luliberin (Camargo et al., 1982; Orlowski et al., 1983) and neurotensin (Camargo et al., 1983; Orlowski et al., 1983). Although the enzyme is not one of the matrix metalloproteinases (Barrett and Tisljar, 1992), there is also evidence for a limited role in the final stages of degradation of the products of collagen breakdown (Morales and Woessner, 1977; Sakyo et al., 1983). Most recently, it has been suggested that thimet oligopeptidase may cleave the Alzheimer's amyloid precursor protein in such a way as to generate amyloidogenic fragments (McDermott et al., 1992).

Knowledge of the properties of thimet oligopeptidase has been based largely on work with the rabbit and rat enzymes (e.g. Carvalho and Camargo, 1981; Orlowski et al., 1989). A recent paper has described some properties of the human enzyme, but without criteria for homogeneity of the preparation (Medeiros et al., 1992). Since the enzyme is thought to be of biomedical importance we felt it was necessary to make a fuller characterization of a homogeneous preparation of human thimet oligopeptidase.

MATERIALS AND METHODS

Materials

Cpp-Ala-Ala-Phe-pAb and Cpp-Ala-Pro-Phe-pAb were prepared as described (Knight and Barrett, 1991). Cpe-Ala-Ala-Phe-pAb was a gift from Dr. Marian Orlowski, Mount Sinai School of Medicine, New York, NY 10029, U.S.A., and Abz-Gly-Gly-Phe-Leu-Arg-Arg-Val-EDDn was given by Dr. Luiz Juliano, Escola Paulista de Medicina, 04034 Sao Paulo, Brazil. Leu-Gly-Gly and Tyr-Gly-Gly were purchased from Sigma.

Thimet oligopeptidase from chicken liver was as described previously (Barrett and Brown, 1990).

Purification of human thimet oligopeptidase

Four packs (1320 ml total) of erythrocytes were obtained from a blood transfusion service. The cells were washed three times by suspension and centrifugation (3500 *g* for 15 min) in 4 litres of 1% (w/v) NaCl containing 0.05% trisodium citrate. The cells were finally resuspended in 2 litres of 10 mM sodium phosphate buffer, pH 7.4, containing 5 mM 2-mercaptoethanol, 0.05% Brij-35 and 25 μM phenylmethanesulphonyl fluoride (buffer A). (Mercaptoethanol and Brij-35 at these concentrations were included in all solutions used subsequently, unless otherwise stated.) The suspension was shaken with 500 ml of carbon tetrachloride, and the emulsion centrifuged (3500 *g*, 15 min). The clear red supernatants were retained, and diluted with 2 litres of buffer A. Whatman DE-52 DEAE-cellulose was equilibrated with buffer A, and 150 g (damp weight) of this was stirred with the lysate for 30 min, by which time essentially all of the thimet oligopeptidase activity (determined as described below) had been adsorbed. The DEAE-cellulose was collected by centrifugation (3500 *g*, 15 min) and washed twice with 2 litres of buffer A in the centrifuge, and further on a sintered glass filter. The adsorbed enzyme was eluted by treating the DEAE-cellulose twice with 250 ml of 500 mM KCl in 100 mM sodium phosphate, pH 7.4, containing Brij-35 and 2-mercaptoethanol as usual in the centrifuge. The two eluates were combined and dialysed against 30 mM Tris/HCl, pH 7.8, containing the usual mercaptoethanol and Brij-35 (buffer B).

The sample was run on a column (200 ml bed vol.) of DEAE-cellulose in buffer B, elution being with a linear gradient to

Abbreviations and conventions used: Abz, *o*-aminobenzoyl; Cpe, *N*-[1(*RS*)-carboxy-3-phenylethyl]; CLIP, adrenocorticotropic hormone fragment 18–39; Cpp, *N*-[1(*RS*)-carboxy-3-phenylpropyl]; Dnp, 2,4-dinitrophenyl; EEDn, *N*-(2,4-dinitrophenyl)ethylenediamine; Mcc, 7-methoxycoumarin-3-carboxyl; pAb, *p*-aminobenzoate; Pz, *N*,α-(4-phenylazo)benzyloxycarbonyl; VIP, vasoactive intestinal peptide (pig, unless otherwise stated). The symbol ↓ is used to indicate the bond cleaved in a substrate, and the terminology of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (1992) is used to number residues either side of the scissile bond.

0.30 M NaCl in 75 mM Tris/HCl buffer, pH 7.8, over 5 bed vol. Fractions containing activity were combined and transferred into 10 mM sodium phosphate buffer, pH 7.0, and concentrated in an Amicon ultrafiltration cell.

The preparation was next run on a column (40 ml bed vol.) of hydroxyapatite (Bio-Rad Bio-Gel HT) in 10 mM sodium phosphate buffer, pH 7.0, with a gradient rising to 150 mM sodium phosphate, pH 7.0, over 6 bed vol. Active fractions were combined and transferred into a buffer containing 20 mM triethanolamine, 10 mM HCl and 200 mM NaCl at pH 7.8, by ultrafiltration, and at the same time concentrated to 2 ml.

The sample was run on a column (92 cm × 16 mm, 185 ml) of Sephacryl S-200 HR (Pharmacia) in the triethanolamine/NaCl buffer. Active fractions were combined, diluted to 100 mM NaCl in the triethanolamine buffer, and run on a Mono Q column (Pharmacia f.p.l.c.) with a gradient to 300 mM NaCl.

The solution was then dialysed into 25 mM Bistris/HCl, pH 6.3, for chromatography on Mono P (Pharmacia f.p.l.c.). Elution was with Polybuffer 74 diluted 10-fold and adjusted to pH 4.0 with HCl. Active fractions were combined to form the final product.

Purification of rat thimet oligopeptidase

Frozen rat testis (100 g) was obtained from a commercial supplier, and homogenized in 200 ml of 10 mM sodium acetate/acetic acid, pH 5.2, at 4 °C. The homogenate was re-adjusted to pH 5.2 with 1 M acetic acid, made to 50 mM total acetate ion, and centrifuged for 30 min at 10000 *g*. The supernatant was adjusted to pH 7.8 with 2 M Tris/HCl, pH 9.0, and diluted with an equal volume of water.

DEAE-cellulose (100 g damp weight) was equilibrated with buffer B and stirred with the preparation for 30 min; it was then collected by filtration, washed with buffer B on the filter, and packed into a column. The elution of the column, and subsequent purification steps were as described for the human enzyme, with two exceptions: the Sephacryl step was omitted, and the pool of active fractions from Mono Q was diluted to 100 mM NaCl in the triethanolamine buffer, and passed through a 1 ml prepacked column of HiTrap Blue (Pharmacia) to remove traces of serum albumin before being run on Mono P.

Enzyme assays

Thimet oligopeptidase was assayed essentially as described by Tisljar et al. (1990), but incubation was at 30 °C rather than 40 °C, and samples were made to a concentration of 1 mM dithiothreitol for 20 min before dilution into the incubation mixture containing 0.2 mM dithiothreitol. The substrate Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) was as supplied by Calbiochem Novabiochem, Nottingham NG7 2QJ, U.K. The Perkin–Elmer fluorimeter was controlled by an IBM-compatible computer running the FLUSYS software (Rawlings and Barrett, 1990). Specific activity of a solution of purified thimet oligopeptidase is expressed as units/$A_{280,1\ cm}$.

Determination of kinetic parameters

Values for K_m were determined by application of the Michaelis–Menten equation, with fitting of the data by non-linear regression analysis (Enzfitter, Elsevier-Biosoft, Cambridge, U.K.). Values for K_i were determined as described by Knight and Barrett (1991), corrected for the effect of substrate on the assumption of simple competition by use of eqn. (1):

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

Determination of rates of hydrolysis of peptides

The rates of hydrolysis of peptides were determined with digestion mixtures containing 100 μM substrate and 1.0 m-unit of enzyme/ml in buffer C. The mixtures were incubated at 37 °C and samples removed at timed intervals, mixed with acetic acid to 1 M and subjected to h.p.l.c. as described above. For each time point the total area for new peaks plus unhydrolysed substrate was calculated and the fraction of intact substrate ($[S]/[S_0]$) remaining was determined. Plots of $\ln([S]/[S_0])$ versus time were linear, so that an apparent first-order rate constant (k) could be calculated according to eqn. (2):

$$\ln([S]/[S_0]) = -kt \quad (2)$$

Identification of cleavage positions in peptides

Cleavage positions were determined for rat thimet oligopeptidase by fractionation of large-scale digests and amino acid analysis of the products. The specificity of the human enzyme was then determined with smaller-scale digests which were run alone as well as mixed with the products of the rat enzyme. Since it was found that the products of the human enzyme always co-chromatographed with those of the rat enzyme, no further identification of products was necessary. In more detail, the methods were as follows.

Before use, solutions of rat or human thimet oligopeptidase solution were mixed with an equal volume of 2 mM dithiothreitol in 500 mM Tris/HCl plus 0.02% Brij, pH 7.8, and allowed to stand for 20 min to allow complete activation of the enzyme. For large-scale digests (2.5 ml), 250 nmol of peptide substrate (100 μM final) was incubated with rat thimet oligopeptidase (2.50 m-unit/ml) at 37 °C in 50 mM Tris/HCl plus 0.02% Brij, pH 7.8, containing 0.2 mM dithiothreitol (buffer C) until 70–90% cleavage was detected by h.p.l.c. (see below). The reaction was stopped with acetic acid to a final concentration of 1 M, and the mixture was subjected to h.p.l.c. on a Varian LC 5000 instrument equipped with the Vista 402 data-processing system and a Techopak 10 C_{18} column (4.6 mm × 250 mm) (HPLC Technology). A linear gradient of 5–70% (v/v) acetonitrile in 0.1% trifluoroacetic acid was run over 25 min at 1 ml/min with monitoring at 220 nm. Peptides were collected and hydrolysed for 16 h at 130 °C in 6 M HCl containing 1% phenol for amino acid analysis (Barrett et al., 1989).

Small-scale digestion mixtures (0.10 ml) contained 50 μM peptide substrate and 0.10 m-unit of enzyme in buffer C. The mixtures were incubated for 60 or 90 min at 37 °C and digestions stopped by the addition of acetic acid to 1 M. Blank assays contained water instead of enzyme. The product mixtures were analysed by h.p.l.c. on a Perkin–Elmer Integral 4000 instrument with a Techopak 10 C_{18} column (4.6 mm × 250 mm). A linear gradient of 5–100% acetonitrile containing 0.1% trifluoroacetic acid was run over 25 min at a flow rate of 1 ml/min with monitoring at 220 nm.

SDS/PAGE and immunoblotting

SDS/PAGE was performed as described by Bury (1981) with 10% (w/v) polyacrylamide gels, and proteins were electroblotted on to a nitrocellulose membrane and immunostained essentially as described by Buttle et al. (1991). The immunoblots were

developed using the adsorbed sheep anti-(rat thimet oligopeptidase) IgG (10 μg/ml) as the primary antibody, biotinylated anti-(sheep IgG) as second antibody and avidin–peroxidase and 4-chloronaphthol for visualization.

Antiserum to rat thimet oligopeptidase

An antiserum was raised in sheep against thimet oligopeptidase from rat testis. The sheep received a total of three intramuscular injections of 100 μg, 100 μg and 50 μg of rat enzyme protein emulsified in Freund's complete adjuvant at zero, 1 and 5 months. Sera from bleeds taken at 7, 10 and 14 days after the third injection were combined. IgG was prepared by triple precipitation from 2.7 M $(NH_4)_2SO_4$, and the final precipitate was dissolved in PBS, pH 7.2, and dialysed against the same. Contaminating antibodies against serum proteins were removed by adsorption with rat serum proteins immobilized on a pre-activated Zetaffinity-10 (Anachem) column.

RESULTS

Purification of human thimet oligopeptidase

The results of the purification procedure are summarized in Table 1 and Figure 1. The initial removal of haemoglobin by batchwise adsorption of the enzyme on DEAE-cellulose effected a 100-fold purification with negligible loss of activity. The progress of purification through the subsequent steps was unexceptional. In contrast with the purification from solid tissues, no difficulty was encountered in preparing the erythrocyte enzyme free of plasma albumin.

The final preparations of both human and rat thimet oligopeptidase ran, in SDS/PAGE, as single major components of M_r about 75000, almost completely free of contaminants (Figure 1a and 1b). The M_r calculated from the deduced amino acid sequence (McKie et al., 1993) is 78308.

The specific activity of the most highly purified human enzyme was about 3 units/mg, but several samples at earlier stages of purification showed higher specific activities (results not shown), and we estimate that the true specific activity of the enzyme is not less than 4 units/mg. The rat enzyme was consistently isolated at 6.5 units/mg. The chicken enzyme purified as described by Barrett and Brown (1990) also showed specific activity of about 6 units/mg under the same conditions of assay.

Figure 1(c) shows an immunoblot of a gel in which the chicken, human and rat enzymes had been run side by side, developed with antiserum raised against the rat enzyme. It can be seen that the human and chicken enzymes, as well as that from rat, were recognized by the antiserum. This broad reactivity of the antiserum across species suggests that the structure of thimet oligopeptidase may have been strongly conserved during evolution.

Thiol- and metal-dependence of activity

The activity of the purified enzyme fell to 13% when it was dialysed for 2 days against 50 mM Tris/HCl buffer, pH 7.8, containing no thiol compound, and assayed without dithiothreitol. Activity was then fully recovered after exposure to 5 mM dithiothreitol for 30 min. Dialysis against 1 mM 1,10-phenanthroline also resulted in complete loss of activity. The human enzyme thus showed the thiol- and metal-dependence characteristic of thimet oligopeptidase from other species (Barrett and Brown, 1990).

Table 1 Purification of human thimet oligopeptidase

The results are those for a typical preparation from 1320 ml of human erythrocytes. 'Lysate' corresponds to the clarified lysate after treatment with carbon tetrachloride. Activity was determined as described in the Materials and methods section, and protein was determined by the Bio-Rad assay (Bradford, 1976) in the first two steps, and then as $A_{280\ nm,1\ cm}$.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification factor	Yield (%)
Lysate	341000	17.0	0.000050	(1)	(100)
DEAE-cellulose batch	3200	16.6	0.00519	104	98
DEAE-cellulose column	415	8.8	0.0212	424	52
Hydroxyapatite	7.6	4.0	0.526	10520	24
Sephacryl S-200 HR	1.4	1.87	1.34	26800	11
Mono Q	0.26	1.0	3.85	77000	6
Mono P (peak fraction)	0.064	0.135	2.11	42200	0.8

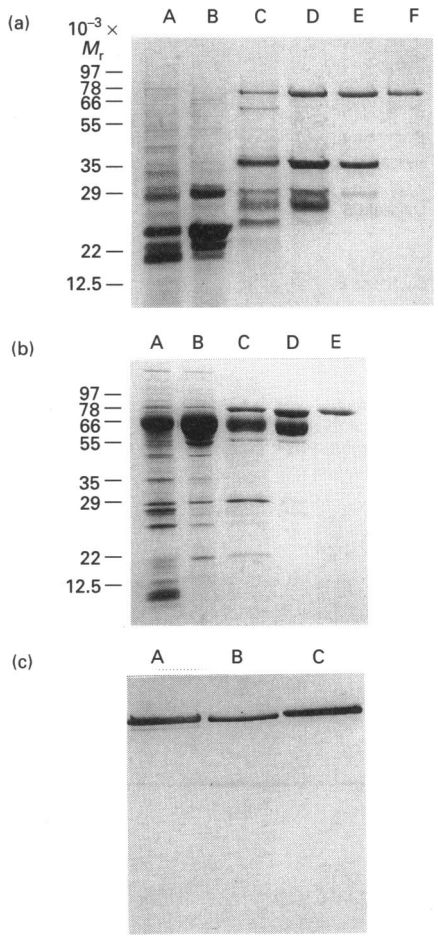

Figure 1 SDS/PAGE of thimet oligopeptidase

(**a**) Progress of purification of the human erythrocyte enzyme. The samples were taken after purification steps as follows: (A) batchwise DEAE-cellulose, (B) DEAE-cellulose column, (C) hydroxyapatite column, (D) Sephacryl S-200 HR, (E) Mono Q f.p.l.c. and (F) Mono P f.p.l.c. (**b**) Purification of the rat testis enzyme. (A) pH 5 supernatant, (B) DEAE- cellulose column, (C) hydroxyapatite column, (D) Mono Q f.p.l.c. and (E) Mono P f.p.l.c. (**c**) Immunoblot of a gel loaded with (A) chicken thimet oligopeptidase, (B) human thimet oligopeptidase and (C) rat thimet oligopeptidase, developed with antiserum against rat thimet oligopeptidase and the detection system described in the Materials and methods section.

Table 2 Points of cleavage of various peptides by thimet oligopeptidase

The names of the peptides are followed by their amino acid sequences in the one-letter code. Points of cleavage were generally determined as described in the Materials and methods section, and all were seen with both human and rat variants of the enzyme. Products containing Dnp, Mcc and pAb were identified by their absorption spectra. Dynorphin A(1–7) and dynorphin A(1–6) were each cleaved into two fragments, one of which was common to both and was identified as Tyr-Gly-Gly by co-elution with an available standard.

		Products isolated	
Peptide	Structure	Components detected	Deduced identity (residues)
Tisljar et al. (1990) substrate	Mcc-P-L+G-P-K(Dnp)	Mcc,P,L	1–2
		G,P,K(Dnp)	3–5
Juliano et al. (1990) substrate	Abz-G-G-F-L+R-R-V-EDDn	G,G,F,L	1–4
		R,R,V,EDDn	5–7
Orlowski et al. (1983) substrate	Bz-G+A-A-F-pAb	G	1
		A,A,F,pAb	2–4
Bradykinin	R-P-P-G-F+S-P-F-R	R,P,P,G,F,	1–5
		S,P,F,R	6–9
Neurotensin	< E-L-Y-E-D-K-P-R+R-P-Y-I-L	E,L,Y,E,D,K,P,R	1–8
		R,P,Y,I,L	9–13
Ac-neurotensin(8–13)	Ac-R+R-P-Y-I-L	R,P,Y,I,L	9–13
Angiotensin I	D-R-V-Y-I-H-P+F-H-L	D,R,V,Y,I,H,P	1–7
		F,H,L	8–10
Angiotensin II	D-R-V-Y+I-H-P-F	D,R,V,Y	1–4
		I,H,P,F	5–8
Dynorphin A(1–8)	Y-G-G-F-L+R-R-I	R,R,I	6–8
		Y-G-G-F-L	1–5
Dynorphin A(1–7)	Y-G-G+F-L-R-R	See legend	
Dynorphin A(1–6)	Y-G-G+F-L-R	See legend	
Dynorphin B	Y-G-G-F-L+R-R-Q-F+K-V-V-T	Y,G,G,F,L	1–5
		R,R,E,F	6–9
Luliberin	< E-H+W-S-Y+G-L-R-P-G-NH_2	H	1–2?
		H,W,S,Y	1–5?
		G,L,R,P,G	6–10
Substance P	R-P-K-P+Q-Q-F+F+G-L-M-NH_2	R,P,K,P	1–4
		E,E,F	5–7
		E,E,F,F	5–8
		F,G,L,M	8–11
		G,L,M	9–11
Neurokinin A	H-L-T-D+S-F-V+G-L-M-NH_2	S,F,V,G,L,M	5–10
		G,L,M	8–10
VIP(1–12)	H-S-D+A-V-F+T-D+N-Y-T-R	H,S,D,A,V,F	1–6
		A,V,F	4–6
		T,D	7–8
		D,Y,T,R	9–12
VIP(16–28) (pig)	< E-M-A-V-K+K-Y-L-N+S-I-L-N-NH_2	E,M,A,V,K	1–5
		K,Y,L,D	6–9
		S,I,L,D	10–13
VIP(16–28) (chicken)	< E-M-A-V-K+K-Y-L-N+S-V-L-T-NH_2	E,M,A,V,K	1–5
		K,Y,L,D	6–9
		S,V,L,T	10–13
α-Endorphin	Y-G-G-F+M+T-S-E-L-S-E+T-P-L-V-T	Y,G,G,F	1–4
		Y,G,G,F,M	1–5
		T,P,L,V,T	12–16

Substrate specificity

Small molecule test substrates

Human thimet oligopeptidase hydrolysed standard synthetic test substrates similarly to the chicken and rat enzymes. Values of K_m for Mcc-Pro-Leu+Gly-Pro-D-Lys(Dnp) were 8.4 μM for the human enzyme and 23 μM for that of rat. Bz-Gly+Ala-Ala-Phe-pAb (Orlowski et al., 1983) was also hydrolysed, as was Abz-Gly-Gly-Phe-Leu+Arg-Arg-Val-EDDn (Juliano et al., 1990). Ac-Ala+Ala-Ala-Ala was cleaved by human thimet oligopeptidase, as it was by the chicken enzyme (Barrett and Brown, 1990). Hexa-alanine was also cleaved, at a similar rate, but unblocked tetra-alanine and penta-alanine were not hydrolysed.

Natural peptides and their derivatives

Other peptides found to be cleaved by human thimet oligopeptidase were bradykinin, substance P, neurokinin A, neurotensin, *N*-acetylneurotensin(8–13), luliberin, deamidated luliberin, dynorphin A(1–6), dynorphin A(1–7), dynorphin A(1–8), dynorphin B, α-endorphin, vasoactive intestinal peptide (VIP) (1–12), VIP(16–28), VIP(chicken, 16–28), angiotensin I, angiotensin II, somatostatin and γ-endorphin. Not detectably

Table 3 Rates of cleavage of peptides by rat thimet oligopeptidase

Each rate, determined as described in the Materials and methods section, is that of the conversion of the intact peptide into fragments, and thus represents the sum of the individual rates of cleavage of each of the scissile bonds in the peptides (the last six in the Table) that were cleaved at more than one point.

Peptide	Initial rate of cleavage (μM/min)
Mcc-P-L-G-P-K(Dnp)	5.4
Abz-G-G-F-L-R-R-V-EDDn	5.4
Bz-Gly-Ala-Ala-Phe-paB	0.7
Bradykinin	6.7
Neurotensin	1.1
Angiotensin I	0.7
Angiotensin II	0.4
Luliberin-NH_2	1.7
Luliberin-COOH	10.1
Substance P	0.6
VIP(1–12)	0.3
VIP(16–28)	1.7
VIP(16–28) (chicken)	0.9

Table 4 Inhibitors of thimet oligopeptidase

The values of the inhibition constant (K_i, corrected for the effect of competition by the substrate) for the rat and human enzymes isolated as described in the present paper are compared with the values for the chicken enzyme reported earlier by Barrett and Brown (1990).

	K_i (nm)		
Compound	Human	Rat	Chicken
Cpe-Ala-Ala-Phe-pAb	397	1100	461
Cpp-Ala-Ala-Phe-pAb	9.8	30	7.5
Cpp-Ala-Pro-Phe-pAb	1.3	7	–
Dynorphin A(1–13)	11.2	48	26

cleaved were Leu-Gly-Gly, neurokinin B, [Leu^5]enkephalin, [Met^5]enkephalin, [D-Ala^6]luliberin, dynorphin A(1–13), dynorphin A(1–17), [D-Trp^{11}]neurotensin, adrenocorticotropic hormone fragment 18–39 (CLIP), VIP(10–28) and intact VIP.

In order to determine points of cleavage of several of the substrates, digestion mixtures were prepared with both rat and human enzymes as described in the Materials and methods section and subjected to h.p.l.c. both singly and mixed. All of the products were formed by both enzymes, and they co-chromatographed in the mixtures. It was notable that all products appeared in parallel, with no indication of further fragmentation of any of them. Many of the products were isolated and identified by amino acid analysis. The 30 cleavage positions that we were able to identify in 19 oligopeptides are shown in Table 2.

Rates of cleavage of peptides

The rates of cleavage of a number of the peptides by the rat enzyme were determined as described in the Materials and methods section, with the results shown in Table 3. These values were used in deciding incubation times for use with the human enzyme in the identification of cleavage sites (above), and in all cases the amounts of hydrolysis were as expected. From this we conclude that the rates of cleavage of these peptides by the human enzyme are approx. similar to those listed for the rat enzyme.

There was a marked difference in the relative rates of degradation of the two forms of luliberin, the free acid form being hydrolysed about tenfold more rapidly than the naturally predominant amide.

Inhibition characteristics

Inhibition constants were determined for human thimet oligopeptidase with carboxyalkyl peptides and dynorphin A(1–13), and the results compared with those for the rat and chicken enzymes (Table 4).

DISCUSSION

A procedure for purification of rat thimet oligopeptidase from testis has previously been described (Orlowski et al., 1989), and we used several similar steps. However, we were able to improve on the final stages in which plasma albumin had been removed by preparative electrophoresis with large losses, by use of affinity chromatography of albumin on HiTrap Blue, in conjunction with f.p.l.c.

The specific activity of human thimet oligopeptidase in erythrocytes was low, but the first purification step, batchwise treatment with DEAE-cellulose, removed haemoglobin, achieving a 100-fold purification with no loss, and subsequently the purification proceeded much as for the rat enzyme. In contrast with the solid tissue sources, persistent contamination by albumin was not seen in the purification from erythrocytes.

The action of the human enzyme on naturally occurring oligopeptides (Table 2) proved to be generally similar to that reported for the enzyme from other species (Cicilini et al., 1988; Toffoletto et al., 1988; Orlowski et al., 1989). We considered the specificity of thimet oligopeptidase for hydrolysis of oligopeptides from three distinct points of view: the effect of substrate size, the location of scissile bonds in relation to free N- and C-termini, and the effects of particular amino acids in the vicinity of the scissile bond.

As has previously been observed for thimet oligopeptidase from other species, the human enzyme is an endopeptidase with substrate-size specificity. We have seen cleavage of unblocked peptides ranging from 6 to 17 residues in length, the largest being γ-endorphin. We did not detect cleavage of Leu-Gly-Gly, reported for the rat mitochondrial enzyme by Heidrich et al. (1973). The substrates of more than 17 amino acid residues that we tested [VIP(10–28), CLIP, VIP] were not cleaved and did not inhibit thimet oligopeptidase activity. The resistance of intact VIP to hydrolysis was particularly striking in view of the fact that the same peptide bonds were readily cleaved in fragments of this peptide. There have been reports of action on larger substrates in the range 18–30 residues (Oliveira et al., 1976; Morales and Woessner, 1977; Dahms and Mentlein, 1992), but having been unable to detect such activity with our own enzyme preparations, we suggest that the earlier reports may have resulted from trace contamination, perhaps by neprilysin (EC 3.4.24.11) or insulinase (EC 3.4.99.45). Since the largest substrate we have found to be cleaved has M_r 1860, we should find any action on the Alzheimer's amyloid precursor protein (M_r 85000), as suggested by McDermott et al. (1992), very surprising.

All the scissile bonds we located are at least three residues into the oligopeptide from a free N- or C-terminus, from which we conclude that unblocked termini are not tolerated within two residues either side of the scissile bond. The cleavage of hexa-alanine, and not of tetra- or penta-alanines, is entirely consistent

with this. However, the cleavages Ac-Ala+Ala-Ala-Ala, < Glu-His+Trp- (in luliberin) and Ac-Arg+Arg- [in *N*-acetylneurotensin(8–13)] show that a blocked N-terminus is accepted closer to the scissile bond. Incidentally, our finding of cleavage of *N*-acetylneurotensin(8–13) is in conflict with the report of Dahms and Mentlein (1992) who reported that this peptide is resistant to thimet oligopeptidase from rat and pig brain.

The requirement for three residues C-terminal to the scissile bond was clearly seen with dynorphin A(1–6) and dynorphin A(1–7), in which the cleavage at Leu+Arg seen in the larger dynorphin fragments is lost, and replaced by the cleavage of Gly+Phe by which neprilysin exerts its 'enkephalinase' activity. The enkephalins were not cleaved, however, presumably being too small (pentapeptides).

There are clear indications that a free C-terminus is favourable in positions P3′, P4′ or P5′. Thus, in all of the peptides for which we have identified scissile bonds, there has been cleavage of a bond three, four or five residues from the C-terminus. This was seen even in peptides with blocked C-termini, but in a number of cases the free C-terminus is clearly beneficial. This was first suggested by Camargo et al. (1982) in reporting that the free-acid form of luliberin (< Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly) is a substrate for the enzyme whereas the naturally occurring amide is not, and that the unnatural amide of bradykinin is less readily hydrolysed than bradykinin itself. Our own observations add to the evidence in support of the importance of the free C-terminus, although we differ in detail from Camargo. Thus we found that the amide form of luliberin was clearly cleaved by both rat and human enzymes [as had been reported by Orlowski et al. (1983) and Medeiros et al. (1992) respectively], but much more slowly than the acid form. Knight and Barrett (1991) noted also the favourable influence of a negative charge at the C-terminus of inhibitors of thimet oligopeptidase, in that K_i for Cpp-Ala-Ala-Phe was 37-fold lower than that for the corresponding amide.

In the h.p.l.c. analysis of samples withdrawn periodically during the hydrolysis of the substrates we studied, there was never any indication that initial products were subsequently further degraded. Even with peptides that were cleaved at more than one point, the peaks arising from the cleavage of each of the susceptible bonds appeared and grew in parallel. None of the primary products was seen to be susceptible to secondary cleavages. From this we conclude that the action of the enzyme is a one-stage process, unlike what is commonly seen with endopeptidases that act on larger polypeptides, and this may be largely attributable to the narrow size range of substrates for the enzyme.

As regards possible selectivity of the enzyme for particular amino acids around the scissile bond, Orlowski et al. (1983) noted that the best substrate in their series contained hydrophobic (Phe) residues in positions P2, P1 and P3′. Subsequently it has been concluded that the enzyme also shows preferences for hydrophobic residues in these positions in natural substrates (Chu and Orlowski, 1985) and in synthetic inhibitors (Orlowski et al., 1988). Now that more data have become available and some of the scissile bonds reported earlier have been corrected, it still seems that the occurrence of hydrophobic residues in P2, P1 and P3′ is more frequent than would be expected by chance. About half of the scissile bonds shown in Table 2 have Ile, Val, Leu or Phe in P3′. The presence of D-Arg in P3′ of Pz-Pro-Leu+Gly-Pro-D-Arg, the standard substrate for 'Pz-peptidase' activity, is an odd exception, particularly in view of the fact that [D-Trp^{11}]neurotensin (with D-Trp in place of Tyr in P3′) was not cleaved.

We suggest that there may be other important determinants of specificity in addition to the hydrophobic character of the P2, P1 and P3′ residues. We have found no occurrence of Val, Leu, Tyr, Pro, His, Asp or Glu in P1′. It seems very likely that all bonds on the imino side of proline are resistant to cleavage by thimet oligopeptidase as they are to many other endopeptidases, but it is notable that prolyl bonds are cleaved. It has been suggested that bonds on the amino side of histidine are also resistant to hydrolysis (Chu and Orlowski, 1985) in view of the odd cleavage of angiotensin II at the Tyr-Ile bond which gives the only occurrence of a very hydrophobic residue (isoleucine) in P1′ and of proline in P3′.

In position P2′ there may well be a significant preference for proline. Proline occurs in this position in two good natural substrates, bradykinin and neurotensin, and in a number of synthetic substrates: Pz-Pro-Leu+Gly-Pro-D-Arg (Barrett and Brown, 1990), Bz-Gly-Phe+Ala-Pro-Phe-pAb and Bz-Gly-Phe+Ser-Pro-Phe-pAb, Dnp-Pro-Leu+Gly-Pro-Trp-D-Lys and an analogue (Barrett et al., 1989; Knight, 1991), and Mcc-Pro-Leu+Gly-Pro-D-Lys(Dnp). In a series of inhibitors, it was shown that a lower K_i value was obtained with proline rather than alanine in the equivalent of P2′ (Knight and Barrett, 1991). A possible reason for the favourable effect of proline in P2′ would be that the residue imposes a preferred conformation on the substrate or inhibitor molecule, as it is suggested to do for some structurally analogous inhibitors of bacterial collagenases (Dive et al., 1991) and substrates of astacin (EC 3.4.24.21) (Stöcker et al., 1990).

We find it difficult to explain the lack of cleavage of neurokinin B (Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2), since the very similar C-terminal sequences in neurokinin A and substance P were susceptible.

With regard to the inhibitors, the potency of the carboxyalkyl inhibitors in the sequence Cpe-Ala-Ala-Phe-pAb < Cpp-Ala-Ala-Phe-pAb < Cpp-Ala-Pro-Phe-pAb for human thimet oligopeptidase is fully consistent with the results for the rat enzyme (Knight and Barrett, 1991). It is notable that the binding constants for the human enzyme are a factor of approx. three lower than for the rat enzyme, however.

Dynorphin A(1–13) has previously been reported to inhibit thimet oligopeptidase of rat (McDermott, 1984), chicken (K_i 26 nM; Barrett and Brown, 1990) and rabbit (K_i 23 nM; Juliano et al., 1990), and is a weaker inhibitor of pitrilysin (K_i 13 μM; Anastasi et al., 1993). Again, the inhibition of human thimet oligopeptidase was more potent than that of the other species.

In conclusion, the indistinguishable patterns of hydrolysis of peptides by thimet oligopeptidase from human, rat and chicken sources, together with the immunological cross-reactivity between the three variants of the enzyme, point to strong conservation of structure and substrate specificity during evolution. The activity of thimet oligopeptidase is to hydrolyse bonds in many peptides in the range six to about 17 residues, which includes many biologically important molecules.

Note added in proof (Received 28 June 1993)

The homogeneous human thimet oligopeptidase (2.5 μg) was run in SDS/PAGE, transblotted on to a poly(vinylidene difluoride) membrane and subjected to N-terminal amino acid sequencing in an Applied Biosystems 477A automated sequencer. A single sequence was obtained: SVVNDL. This hexapeptide, occurs only once in the Swissprot database (Rel. 25.0) for regulatory protein sir3 of yeast. More significantly, however, it occurs in the sequence of an enzyme described as 'amyloidin' (Dovey et al., 1992) which has 88% identity over the complete sequence (688

residues) to the corrected sequence of rat thimet oligopeptidase (McKie et al., 1993). The N-terminus of this is: MKPPAACAGDMADAASPCSVVNDLRWD... The enzymological properties described for amyloidin are closely similar to those of thimet oligopeptidase, and it seems safe to conclude that amyloidin is human thimet oligopeptidase. Dovey et al. found the N-terminus of amyloidin to be blocked. The significance of our isolation of a form of the protein lacking the N-terminal 18 residues remains to be investigated.

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