Thimet oligopeptidase: similarity to 'soluble angiotensin II-binding protein' and some corrections to the published amino acid sequence of the rat testis enzyme

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The deduced amino acid sequence of pig liver soluble angiotensin II-binding protein [Sugiura, Hagiwara and Hirose (1992) J. Biol. Chem. **267**, 18067–18072] is similar over most of its length to that reported for rat testis thimet oligopeptidase (EC 3.4.24.15) by Pierotti, Dong, Glucksman, Orlowski and Roberts [(1990) (Biochemistry **29**, 10323–10329]. We have found that homogeneous rat testis thimet oligopeptidase binds angiotensin II with the same distinctive characteristics as the pig liver protein. Analysis of the nucleotide sequences reported for the two proteins pointed to the likelihood that sequencing errors had caused two segments of the amino acid sequence of the rat protein to be translated out of frame, and re-sequencing of selected parts of the clone (kindly provided by the previous authors) confirmed this. The revised deduced amino acid sequence of rat thimet oligopeptidase contains 687 residues, representing a protein of 78308 Da, and is more closely related to those of the pig liver protein and other known homologues of thimet oligopeptidase than that described previously.

INTRODUCTION

Thimet oligopeptidase (EC 3.4.24.15) (also known as soluble metalloendopeptidase) is a thiol-dependent metalloendopeptidase with the unusual characteristic of acting only on substrates of less than about 17 amino acid residues (Dando et al., 1993). This suggests that its physiological function may be the generation or degradation of 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). Thimet oligopeptidase also acts on angiotensin I and angiotensin II (Chu and Orlowski, 1985; Dando et al., 1993). Thimet oligopeptidase is a primarily cytosolic enzyme, but is reported to have a minor membrane-associated component (Acker et al., 1987). A deduced amino acid sequence of thimet oligopeptidase from rat testis reported by Pierotti et al. (1990) is that of a protein of 73 kDa.

Work aimed at the isolation of the angiotensin II receptor led Kiron and Soffer (1989) to purify an angiotensin II-binding protein from rabbit liver. This protein, of 75 kDa, was predominantly cytosolic, with a 5% membrane-bound component. The protein was simultaneously isolated from pig liver by Hagiwara et al. (1989), and was cloned and sequenced from a pig heart library (Sugiura et al., 1992).

We have noticed similarities between the amino acid sequences reported for thimet oligopeptidase and soluble angiotensin II-binding protein, and have investigated the implications of these.

MATERIALS AND METHODS

Materials

Thimet oligopeptidase was purified from rat testis as described by Dando et al. (1993). [3-[^{125}I]Iodotyrosyl^{4}]-angiotensin II(5-L-isoleucine) (about 74 TBq/mmol or 2000 Ci/mmol) was code IM 177 from Amersham.

A cDNA clone 50 with a 2.2 kb insert encoding the open reading frame of thimet oligopeptidase was generously given by Dr. J. L. Roberts and Dr. M. Orlowski, Mount Sinai School of Medicine, NY, U.S.A.

Assay of angiotensin II binding

This was essentially as described by Hagiwara et al. (1989), except for the inclusion of detergent in the assay buffer following Sen et al. (1983). The assay buffer was 30 mM Tris/HCl, pH 7.5, containing 2.5 mM EDTA, 0.8 mM *p*-chloromercuriphenylsulphonate, 0.25% Brij 99 and 0.1% BSA. Each complete reaction mixture (150 μl) contained 0.1 nM ^{125}I-labelled angiotensin II (about 90000 c.p.m.), 20 nM unlabelled angiotensin II and 4 μg of purified rat thimet oligopeptidase in assay buffer. The mixture was incubated for 45 min at 30 °C, and then treated with 0.5 ml of 0.05% Dextran 500 (Sigma)/0.5% Norit-A charcoal (Aldrich) for 10 min at 4 °C, and centrifuged in a microfuge also for 10 min at 4 °C. The supernatant was sampled for counting of protein-bound radiolabel. Negative controls for non-specific binding contained 10 μM unlabelled angiotensin II or no enzyme.

Cross-linking of radiolabelled angiotensin II to thimet oligopeptidase

Reaction mixtures (300 μl) contained assay buffer as for the standard binding assay, radiolabelled angiotensin II (1×10^6 c.p.m.), 10 μg of rat testis thimet oligopeptidase and either 0 or 10 μM unlabelled angiotensin II. After 45 min at 30 °C the mixtures were treated with 900 μl of dextran/charcoal for 10 min at 4 °C before being centrifuged. Cross-linking of the bound ^{125}I-labelled angiotensin II was achieved by treating the supernatants with 1 mM disuccinimidyl suberate (Pierce) for 15 min at room temperature. The reaction was stopped by adding ammonium acetate to a final concentration of 100 mM. The samples containing cross-linked complexes of rat thimet

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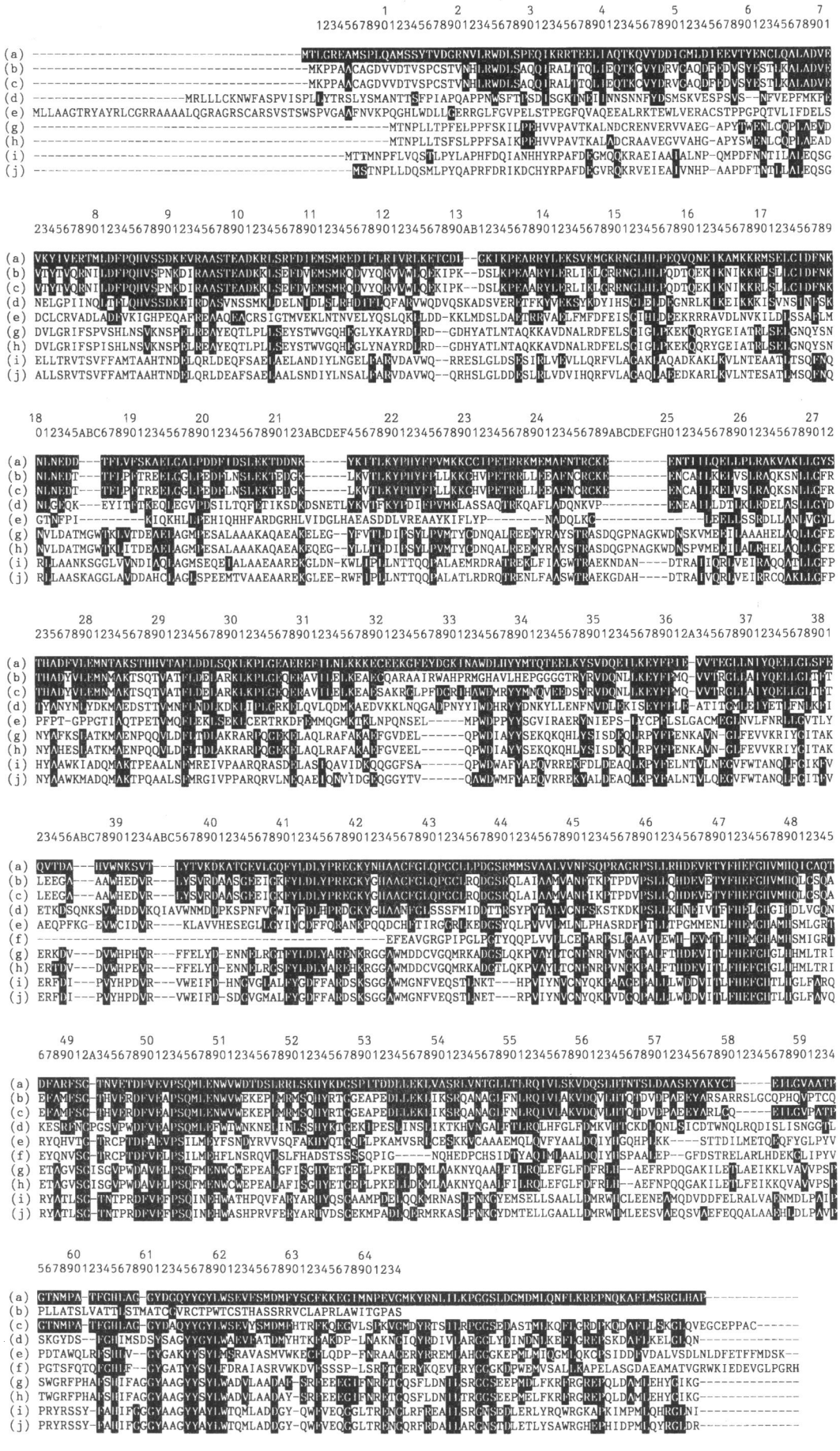

oligopeptidase and ^{125}I-labelled angiotensin II were concentrated by precipitation with trichloroacetic acid (10%, w/v) before being run on SDS/10%-polyacrylamide gels under reducing conditions. After electrophoresis the gels were dried and exposed to Fuji RX medical X-ray film for 75 h at −70 °C with the use of an intensifying screen.

Nucleotide sequence determination

Restriction fragments derived from the thimet oligopeptidase open reading frame by digestion with *Acc*I were subcloned by standard procedures (Sambrook et al., 1989) into M13mp18 and M13mp19 (Messing, 1983) or alternatively into the plasmid pTZ18R (Pharmacia). DNA was sequenced by the di-deoxy chain-termination method (Sanger et al., 1977) with universal primer by use of the enzyme Sequenase (U.S.B., Cleveland, OH, U.S.A.) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

An alignment of the sequence reported for rat testis thimet oligopeptidase (Pierotti et al., 1990) with that of the pig liver soluble angiotensin II-binding protein (Sugiura et al., 1992) (Figure 1, sequences a and b) indicated that the proteins are closely related, perhaps being species variants of a single protein.

Binding of angiotensin II by thimet oligopeptidase

The soluble angiotensin II-binding protein binds angiotensin with high affinity, but only under non-physiological conditions, i.e. in the presence of EDTA and of *p*-chloromercuriphenylsulphonate (Sen et al., 1983; Hagiwara et al., 1989). Accordingly, we examined homogeneous rat thimet oligopeptidase for angiotensin II-binding activity in the assay that had been used for the angiotensin II-binding protein. As shown in Table 1, we found that thimet oligopeptidase bound radiolabelled angiotensin II, and the binding was blocked by unlabelled angiotensin II. Binding was strongly dependent on the presence of EDTA and *p*-chloromercuriphenylsulphonate, and could be partially suppressed by omission of the Brij 99 detergent or inclusion of dithiothreitol. It thus showed the distinctive characteristics of binding by the angiotensin II-binding protein.

Angiotensin II bound by soluble angiotensin-binding protein can be covalently cross-linked to the protein with disuccinimidyl suberate (Hagiwara et al., 1989). We used the same method (see the Materials and methods section) with thimet oligopeptidase. The 1 ml supernatants after removal of free radioactivity contained 6.47×10^4 and 4.6×10^3 c.p.m., respectively, in the absence or presence of excess unlabelled angiotensin II. The autoradiograph of the SDS/PAGE gel showed a radioactive band in the position at which thimet oligopeptidase was detected by protein staining and immuno-blot detection (results not shown).

Table 1 Binding of radiolabelled angiotensin II by rat thimet oligopeptidase

Assay mixtures containing 4 μg of rat thimet oligopeptidase were set up as described in the Materials and methods section, and values are means of duplicates expressed as percentages of the result for the complete system (6705 c.p.m.). The experiment with dithiothreitol was done by addition of the thiol compound after the standard 45 min incubation period, allowing a further 10 min before removal of unbound angiotensin with charcoal.

System	Binding (%)
Complete	(100)
− Rat thimet oligopeptidase	5.8
+ Angiotensin II (10 μM)	12.0
− EDTA	14.6
− *p*-Chloromercuriphenylsulphonate	8.7
− Brij 99	54.2
+ Dithiothreitol (5 mM)	22.8

Re-sequencing of selected parts of the cDNA

The clone (50) given to us by Roberts and Orlowski was first characterized by restriction-enzyme digestion. It was cleaved as expected by the enzymes *Eco*RI, *Pst*I, *Kpn*I and *Acc*I, and gave expression in transformed *Escherichia coli* of thimet oligopeptidase that was active on 7-methoxycoumarin-3-carboxylyl-Pro-Leu-Gly-Pro-D-Lys(Dnp) (Tisljar et al., 1990) and reacted with antisera to the rat enzyme (Dando et al., 1993). However, the insert was not cleaved by *Xho*I, contrary to restriction-mapping data presented by Pierotti et al. (1990). (The numbering system used for nucleotides and amino acids throughout the present paper is that of Pierotti et al., 1990.)

The alignment of the published amino acid sequences of thimet oligopeptidase and the pig liver protein (Fig. 1) contains the segments 321–347 and 577 to the C-terminus in which very few identities occur. The nucleotide sequence corresponding to the segment 297–350 was accordingly re-determined. The result differed from that of Pierotti et al. (1990) in that two additional bases, C-G, were found between 1018 and 1019, and the bases 1096 (A) and 1099 (C) previously reported were not found. This has the effect of changing the deduced amino acid sequence in the segment 320–347 (Figure 1).

The impression gained from the alignment that a further error may have occurred from about residue 577 to the C-terminus was supported by the results of computer-assisted analysis of the nucleotide sequence given for rat testis thimet oligopeptidase (Pierotti et al., 1990) with the NIP program (Staden, 1990), which pointed to a reading-frame error in this part of the molecule. Again, from re-sequencing of the appropriate subclone, an additional base (G) was found between 1790 and 1791. This change affects the deduced sequence for amino acids 577 to the C-terminus (Figure 1).

The revised deduced amino acid sequence for the open reading frame contains 687 residues, as opposed to 645 reported pre-

Figure 1 Alignment of the sequence of pig liver angiotensin II-binding protein with the previous and revised sequences of rat testis thimet oligopeptidase, and those of other homologous proteins

The deduced amino acid sequence of (a) pig liver soluble angiotensin II-binding protein (Sugiura et al., 1992) is aligned with (b) that previously reported for rat testis thimet oligopeptidase (Pierotti et al., 1990) and (c) the revised sequence of rat thimet oligopeptidase described here. The sequences of other members of the family are (d) saccharolysin (EC 3.4.24.37, described as open reading frame YCL57w) (Oliver et al., 1992), (e) rat mitochondrial intermediate peptidase (Isaya et al., 1992), (f) *Schizophyllum commune* putative metalloendopeptidase (Giasson et al., 1989), (g) *Escherichia coli* oligopeptidase A (Conlin et al., 1992), (h) *Salmonella typhimurium* oligopeptidase A (Conlin and Miller, 1992), (i) *E. coli* dipeptidyl carboxypeptidase (S. Becker and R. Plapp, Swissprot database entry DCP_ECOLI), and (j) *S. typhimurium* dipeptidyl carboxypeptidase (Hamilton and Miller, 1992). Residues printed in white on black are identical with those in soluble angiotensin II-binding protein.

viously, and the calculated molecular mass of the polypeptide (assuming no disulphide bridges) is 78308 Da rather than 73000 Da. The rat and pig proteins contain 62% identical residues. As can be seen in Figure 1, the revised sequence also shows increased similarity to those of other proteins that are members of the thimet oligopeptidase family (Rawlings and Barrett, 1993), for which the percentage identities are 20–36%.

We conclude that rat testis thimet oligopeptidase and pig liver soluble angiotensin II-binding protein are more closely related to each other than to other members of their family of proteins, and also resemble each other in angiotensin II-binding activity. There is no evidence as to whether the pig liver angiotensin II-binding protein has the enzymic activity of thimet oligopeptidase, but the possibility must exist that the sequence reported by Sugiura et al. (1992) is that of pig thimet oligopeptidase. However, the binding of angiotensin II by the protein is probably unrelated to its enzymic activity, since the binding is dependent on the presence of EDTA and *p*-chloromercuriphenylsulphonate (Hagiwara et al., 1989; Kiron and Soffer, 1989), both of which are inhibitory to thimet oligopeptidase (Barrett and Brown, 1990).

Note added in proof (received 4 August 1993)

Since the present paper was submitted, the deduced amino acid sequence of a putative processing endopeptidase from rabbit liver has been reported (Kawabata et al., 1993). This sheds new light on the question of whether or not soluble angiotensin II-binding protein corresponds to a species variant of thimet oligopeptidase. The rabbit protein is 91% identical in sequence with the pig angiotensin-binding protein, in contrast with the values of 61% and 62%, respectively, for the comparison of rat thimet oligopeptidase with rabbit processing endopeptidase and pig angiotensin-binding protein. In view of this, it seems most probable that the pig protein corresponds to a species variant of the putative processing enzyme, rather than thimet oligopeptidase.

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