Dipeptidyl peptidase III is a zinc metallo-exopeptidase

Molecular cloning and expression

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We have purified dipeptidyl peptidase III (EC 3.4.14.4) from human placenta. It had a pH optimum of 8.8 and readily hydrolysed Arg-Arg-β-naphthylamide. Monoamino acid-, Gly-Phe-, Gly-Pro- and Bz-Arg-β-naphthylamides were not hydrolysed at all. The enzyme was inhibited by *p*-chloromercuriphenylsulphonic acid, metal chelators and 3,4-dichloroisocoumarin and contained 1 mol of zinc per mol of enzyme. The zinc dissociation constant was 250 fM at pH 7.4 as determined by the zinc binding study. We isolated, by immunological screening of a Uni-ZAP XR cDNA library constructed from rat liver mRNA species, a cDNA clone with 2633 bp encoding the rat enzyme. The longest open reading frame encodes a 827-residue protein with a theoretical molecular mass of 92790 Da. *Escherichia coli* SOLR cells were infected with the pBluescript phagemid containing the cloned cDNA and established the overexpression of a protein that hydrolysed Arg-Arg-β-naphthylamide. The recombinant protein was purified and the amino acid sequence of the protein was confirmed. We presumed that the putative zinc-binding domain involved in catalysis was present in the recombinant enzyme. It was a novel zinc-binding motif in that one amino acid residue was inserted into the conserved HEXXH motif characteristic of the metalloproteinases.

INTRODUCTION

Dipeptidyl peptidase (DPP) III, discovered by Ellis and Nuenke [1] in the bovine anterior pituitary, cleaves Arg-Arg-NA (where NA stands for β-naphthylamide) with a pH optimum of 9.0 and is inhibited by thiol reagents. The enzyme from rat brain cytosol prefers angiotensins and enkephalins as substrates at pH 7.4 and is inhibited not only by thiol reagents but also by the metal-chelating agent *o*-phenanthroline [2]. DPP III has been demonstrated to be a metalloenzyme by inhibition and restoration experiments on the activity utilizing metal chelators and metal ions [3,4]. However, DPP III from human cataractous lens [5] and pig spleen [6] was also inhibited by di-isopropyl fluorophosphate (DFP) as well as by metal-chelating agents.

Here we demonstrate, by an atomic absorption experiment, that DPP III contains a Zn^{2+} ion and, by a zinc binding study, showed that the enzyme has only one Zn^{2+} ion in its active site. Based on the data we propose DPP III to be a metalloenzyme with a novel zinc-binding motif.

EXPERIMENTAL

Materials

Various aminoacyl-NAs (Arg-, Ala-, Leu-, Pro-, Gly-Phe-, Lys-Ala-, Arg-Arg-), *p*-chloromercuriphenylsulphonic acid (PCMPS), 3,4-dichloroisocoumarin (DCI) and Fast Garnet GBC were obtained from Sigma. Lys-NA came from Koch-Light (Colnbrook, Bucks., U.K.); and Gly-Pro-NA, from Backem Feinchemikalien (Bubendorf, Switzerland). Restriction and modifying enzymes were from Toyobo Co. (Osaka, Japan) and Takara Co. (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Nakarai Tesque (Kyoto, Japan) and Daiichi Chemicals (Tokyo, Japan).

Protein and enzyme assay

Protein was usually measured by the method of Hartree [7]. However, when the sample solution contained a reducing agent the protein concentration was measured by the method of Ross and Schatz [8]; if the sample solution contained a micro quantity of protein, the method of Sargent [9] was used, with BSA as the standard.

For the standard assay buffer, 50 mM sodium phosphate buffer, pH 7.4, was used, as described by Lee and Snyder [2]. The incubation mixture contained 25 mM buffer, 0.2 mM Arg-Arg-NA and an appropriate amount of enzyme plus water to 200 μl. After incubation at 37 °C for 20 min, 500 μl of 1 M acetate buffer, pH 4.0, containing 10 % (v/v) Tween-20 and 200 μl of Fast Garnet GBC (0.2 mg/ml in water) were added to the reaction mixture. The absorbance of the resulting diazo dye was measured at 530 nm. When the reaction mixture contained a reducing agent the reaction was stopped by adding 100 μl of 40 % (w/v) trichloroacetic acid; the amount of β-naphthylamine liberated was then estimated by the method of Goldbarg and Rutenburg [10].

The DPP III activity during purification (steps 1–3) was assayed in the presence of 0.02 μmol of bestatin to inhibit the aminopeptidase activity. One unit of enzyme activity is defined as the amount catalysing the formation of 1 μmol of β-naphthylamine/min at 37 °C.

Purification of enzyme from human placenta and production of the antibody

Normal-term human placentas were collected immediately after delivery and kept on ice before homogenization. The fetal membranes and umbilical cord were dissected away; each

Abbreviations used: DCI, 3,4-dichloroisocoumarin; DFP, di-isopropyl fluorophosphate; DPP, dipeptidyl peptidase; NA, β-naphthylamide; 2,6-PA, 2,6-pyridinedicarboxylate; PCMPS, *p*-chloromercuriphenylsulphonic acid.

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The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D89340.

placenta was washed free from blood with cold 0.9 % NaCl and cut into small pieces (496 g wet weight). The tissue was then homogenized in 8 vol. of 0.9 % NaCl with an Ultra-Turrax homogenizer (20000 rev./min, 3 min). The homogenate was centrifuged at 10000 ***g*** for 30 min, and the resulting supernatant was centrifuged at 55000 ***g*** for 60 min. The supernatant fluid was fractionated by $(NH_4)_2SO_4$ precipitation (40–65 % satn.) with solid $(NH_4)_2SO_4$ (step 1; see Table 1). All of the following purification steps were performed at 4 °C unless otherwise specified. Each step used sodium phosphate buffer [10 mM sodium phosphate, pH 8.0, containing 50 mM NaCl (buffer A) or 1 mM sodium phosphate, pH 6.8, containing 5 mM 2-mercaptoethanol (buffer B)]. The $(NH_4)_2SO_4$ fraction was dialysed against buffer A and then applied to a DEAE-Toyo-column (4.7 cm × 25 cm) that had been equilibrated with the same buffer. The column was washed with 2 vol. of the starting buffer and then eluted with a linear gradient established between 700 ml of buffer A and 700 ml of the same buffer containing 0.4 M NaCl.

Fractions of 9.5 ml were collected (active fraction numbers 59–68; step 2). The pooled enzyme solution was dialysed against buffer B and concentrated in collodion bags to 10 ml. The concentrated material was then applied to a hydroxyapatite column (2.8 cm × 25 cm) equilibrated with buffer B and eluted with a linear phosphate gradient from 1 to 100 mM in buffer B (volumes 500 ml and 500 ml; 8.5 ml per tube; active fraction numbers 41–50; step 3). Active fractions were concentrated in a collodion bag to 6 ml, and solid $(NH_4)_2SO_4$ (25 % satn.) was then added to it. The $(NH_4)_2SO_4$ solution was applied to a phenyl-Sepharose column (1.6 cm × 16 cm) and eluted with a double linear gradient [$(NH_4)_2SO_4$ concentration 25–0 %; ethylene glycol 0–50 %] in the buffer (volumes 100 ml and 100 ml; 3.5 ml per tube; active fraction numbers 48–52; step 4). Active fractions were combined, dialysed against buffer A, concentrated in a collodion bag to 1 ml and then loaded on a Sephacryl S-300 column (2.6 cm × 95 cm; 8.73 ml per tube; active fraction numbers 39–43; step 5; see Table 1 and Figure 2A). Enzyme fractions were pooled and concentrated to 0.292 ml. This enzyme preparation was mixed with an equal volume of glycerol and stored at −20 °C until used for immunization.

The purified enzyme (150 μg/ml) was emulsified with 1 ml of complete Freund's adjuvant and injected subcutaneously into a 2 kg male rabbit. After 10 days, 75 μg of the antigen was injected three times at 2 week intervals in the same manner; 10 days after the last injection the animal was bled from the carotid artery. The selective reactivity between purified human placental enzyme and its antiserum was verified by the double-immunodiffusion technique and Western blot analysis. The antiserum was stored at −80 °C.

Electrophoresis was performed under denaturing and reducing conditions on 10.0 % (w/v) polyacrylamide gels by the method of Laemmli [11]. The proteins were stained with Coomassie Blue R-250 and then blotted on nitrocellulose membranes (Bio-Rad) in a Bio-Rad apparatus (2.5 h, 40 V). The membranes were blocked at room temperature in PBS, pH 7.5, containing 3 % (w/v) BSA and 0.05 % Tween-20. Rabbit anti-human DPP III antiserum was diluted 1:3000 with the same buffer, and the membranes were incubated in it overnight at 4 °C. The membranes were then washed three times in PBS containing 0.05 % Tween-20 and incubated for 1 h with alkaline phosphatase-conjugated goat anti-(rabbit IgG) (Bio-Rad) diluted 1:2000 with blocking solution. After the membranes had been washed three times with PBS containing 0.05 % Tween-20, the bands were revealed by use of an alkaline phosphatase colour development kit (Bio-Rad).

Properties of the purified enzymes

K_m values were deduced from Lineweaver–Burk plots by a linear regression program (mean ± S.D.). The amount of substrates used in these kinetic studies ranged from 0.01 to 0.05 μmol. Amounts of enzymes tested were as follows: placental enzyme, 0.063 μg (for Arg-Arg-NA) and 0.209 μg (for Lys-Ala-NA); recombinant enzyme, 0.244 μg (for Arg-Arg-NA) and 1.71 μg (for Lys-Ala-NA).

k_{cat} values were calculated for a theoretical molecular mass of 92790 Da for both enzymes because their mobilities relative to those of standard marker proteins by SDS/PAGE and Sephacryl-S-300 column chromatography were almost identical. Marker proteins (Bio-Rad) and their R_F values (mean) on an SDS/10 % (w/v) polyacrylamide gel were as follows: myosin (200 kDa, 0.065), *E. coli* β-galactosidase (116.25 kDa, 0.145_6), rabbit muscle phosphorylase *b* (97.4 kDa, 0.186), BSA (66.2 kDa, 0.269_3), hen egg-white ovalbumin (45 kDa, 0.436_6), bovine carbonic anhydrase (31 kDa, 0.633_3) and hen egg-white lysozyme (21.5 kDa, 0.849). SDS/PAGE for molecular mass determination was done four times for both enzymes on SDS/10 % (w/v) polyacrylamide gels; the following R_F values were obtained: 0.232, 0.236, 0.240 and 0.248. The molecular mass value was calculated by a logarithmic regression program (mean ± S.D.; see Table 2).

The molecular masses of markers (Pharmacia Biotech) and their fraction numbers on the Sephacryl-S-300 (2.6 cm × 95 cm) column (elution buffer, buffer A; fraction volume, 1 ml) were as follows: Blue Dextran 2000 (2000 kDa, 235); catalase (232 kDa, 335); aldolase (158 kDa, 341); BSA (67 kDa, 364); ovalbumin (43 kDa, 381). Both enzymes were loaded on the column for three separate elutions, and the fraction numbers for the peak activity were 357, 358 and 358. The value for the molecular mass was calculated by a logarithmic regression program (mean ± S.D.; see Table 2).

Inhibitors were preincubated for 10 min at 37 °C with the enzyme (0.063 μg). The reaction was initiated by the addition of 0.02 μmol of Arg-Arg-NA. All inhibitors were prepared as stock solutions in the appropriate solvent [12]: DMSO for DCI, PCMPS and *o*-phenanthroline; dry propan-2-ol for PMSF and DFP; and water for EDTA, *N*-ethylmaleimide and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64). Each final solvent concentration was held constant at 10 % (v/v) (DMSO) or 1 % (v/v) (propan-2-ol). Results were expressed as the concentration giving 50 % inhibition of the control activity (calculated by a logarithmic regression program; mean ± S.D.; see Table 3). Zinc determination was performed by electrothermal atomic absorption spectrometry with a Zeeman atomic absorption spectrometer SM-30 (Shimazu Co., Tokyo, Japan). Absorbance peak heights were measured at 370.6 nm. For the zinc determination the enzymes were applied to a Nick column (containing Sephadex G-50; Pharmacia) and eluted with distilled water. Then the eluted solution was divided into two parts for the analysis of zinc and determination of amino acid composition. The sample for zinc analysis was freeze–dried, dissolved in 0.01 % (v/v) metal-free nitric acid and diluted appropriately. Samples (10 μl) were loaded into pyrolytically coated graphite tubes. The values shown are averages of three different dilutions of sample. Zinc concentrations were quantified by comparing unknowns with zinc standard solutions (0, 50, 75 and 100 ng; Wako Pure Chemical Industry). The amino acid composition of the sample described above was determined after hydrolysis under vacuum in HCl vapour at 105 °C for 24, 48 and 72 h, followed by precolumn derivatization with phenylisothiocyanate (Waters Associates; PICO-TAG method) and reverse-phase HPLC.

The following buffers were substituted for the standard assay buffer for determination of the pH activity profile: the human placental enzyme was incubated with 10 μM borate buffer (pH 8.0–10.0), and the recombinant enzyme with 10 μM Tris/HCl buffer (pH 7.0–9.0). Results were expressed as the percentage of maximum activity (results are mean values of three determinations; see Figure 3).

Zinc binding studies

The simple technique to estimate the zinc dissociation constant was based on the measurement of the enzyme activity in a series of metal buffer systems with known concentrations of free Zn^{2+} ions. The metal buffers were solutions of the Zn^{2+} ions and excess chelating agent [2,6-pyridinedicarboxylate (2,6-PA)]. The available concentration of the metal in such a solution depends on the stability constants of the significant chelates, on the concentration ratio of metal to chelator, and on pH. The corresponding calculations, based on data from conditional stability constants of 2,6-PA at pH 7.4 ($\beta_1' = 10^{7.0}$ and $\beta_2' = 10^{13.0}$ [13]), were performed with the aid of a basic computer program. The coordination efficiencies of phosphate, OH^- and Cl^- ions in the buffer solution are very low and were not taken into account. The chelating agent used was 2,6-PA, which is suitable for controlling Zn^{2+} concentrations between 250 pM and 4.0 fM at pH 7.4.

The concentration of free Zn^{2+} ions ($[Zn^{2+}]$) was calculated by the following equation with the total concentration of 2,6-PA (L_T), the total zinc concentration (Zn_T) and the conditional stability constants of 2,6-PA (β_1' and β_2'):

$$[Zn^{2+}] = \frac{[Zn^{2+}]+[ZnL]+[ZnL_2]}{1+\beta_1'[L]+\beta_2'[L]^2} = \frac{Zn_T}{1+\beta_1'[L]+\beta_2'[L]^2} \quad (1)$$

$$L_T = [L]+\beta_1'[L][Zn^{2+}]+2\beta_2'[L]^2[Zn^{2+}] = [L]+[Zn^{2+}](\beta_1'[L]+2\beta_2'[L]^2) \quad (2)$$

where [L], [ZnL] and $[ZnL_2]$ are the free 2,6-PA concentration, the concentration of a 1:1 zinc/2,6-PA complex and the concentration of a 1:2 zinc/2,6-PA complex respectively [14].

Insertion of eqn. (1) into eqn. (2) gives:

$$L_T = [L]+\frac{Zn_T}{1+\beta_1'[L]+\beta_2'[L]^2}\cdot(\beta_1'[L]+2\beta_2'[L]^2) \quad (3)$$

In eqn. (3) it is necessary to solve a third-order equation to determine the free 2,6-PA concentration, [L]. Therefore [L] was determined by a computation minimizing the square deviation, U, given by:

$$U = (L_{T(calc)}-L_T)^2 \quad (4)$$

where $L_{T(calc)}$ is the calculated total concentration of 2,6-PA at a given [L] with eqn. (3) [14,15]. Computation was performed on an NEC PC-9801 RA programmable calculator with a pit-mapping program [14]. The concentration of free Zn^{2+} ions ($[Zn^{2+}]$) was determined by inserting the concentration of free 2,6-PA ([L]) obtained by eqns. (3) and (4) into eqn. (1).

The dissociation of the Zn^{2+} ions from DPP III is described by:

$$EZn \rightleftharpoons E + Zn^{2+} \quad (5)$$

$$K_d = ([E][Zn^{2+}])/[EZn] \quad (6)$$

where EZn, E and K_d are DPP III, apo-DPP III and the dissociation constant of the Zn^{2+} ions from DPP III respectively.

The Zn^{2+} ion in DPP III is essential for enzyme activity. In the addition of the metal buffer to the enzyme, the residual activity of the enzyme is shown by

$$\text{Residual activity } (\%) = 100[EZn]/([EZn]+[E]) = 100[Zn^{2+}]/(K_d+[Zn^{2+}]) \quad (7)$$

where the free Zn^{2+} ions in the metal buffer is calculated from eqn. (1).

Cloning and sequencing of the rat DPP III cDNA

For isolation of rat liver cDNA, mRNA from rat (female Wistar) liver was obtained by use of a mRNA isolation kit; then the cDNA was constructed with a Uni-ZAP synthesis kit from Stratagene. From this library, 5.0×10^5 plaques were screened immunologically with polyclonal rabbit antiserum against the human placental DPP III. pBluescript phagemids containing cDNA inserts between *Eco*RI and *Xho*I sites were excised from the Uni-ZAP clones by the use of ExAssist helper phage. VCSM13 helper phages were used for the single-stranded rescue procedure.

The nucleotide sequence was determined by the dideoxynucleotide chain-termination reaction method [16]. Restriction endonuclease fragments of the cDNA were subcloned into M13 mp 18 or mp 19, and all regions of these single-stranded DNA species were sequenced. Also, the single-stranded pBluescript SK(−) phagemid containing full-length cDNA was partly sequenced to fill in remaining gaps in the sequence with the specific primers. Specific oligomers were purchased from Sawaday Co. (Tokyo, Japan). Figure 1 shows the restriction map and sequence strategy.

Expression of recombinant enzyme and its tryptic peptide sequencing

Cultures of *Escherichia coli* SOLR cells infected with the recombinant pBluescript phagemid were grown at 37 °C to $D_{600} = 0.2$ in 3 litres of Luria–Bertani medium containing 150 mg of ampicillin. Then isopropyl β-D-thiogalactoside was added to a final concentration of 1.0 mM; the bacteria were incubated overnight. After centrifugation at 10000 ***g*** for 15 min, the cells were suspended in 270 ml of 0.9 % NaCl and disrupted by ultrasonic treatment at 9 kHz for 1 min, which was repeated three times at intervals of 5 min. A clear supernatant fluid was obtained by centrifugation at 25000 ***g*** for 30 min, to which solid $(NH_4)_2SO_4$ was added (65 % satn.; step 1). Column chromatographic procedures were the same for the purification of the human placental enzyme. The recombinant enzyme (1.48 mg) was obtained, the specific activity of which corresponded to a 62-fold purification from step 1 with a recovery of 16 % (see Figure 2B).

Recombinant enzyme was digested with 2 % (w/w) trypsin in 100 mM ammonium bicarbonate buffer, pH 8.0, for 16 h at 37 °C. The digest was fractionated on a cation-exchange HPLC column (Pepkat 300; 5 μm, 4.0 mm × 125 mm) with 5 mM phosphate buffer, pH 3.0, and a linear gradient from 0 to 0.5 M NaCl for 30 min at a flow rate of 1 ml/min. Ten major peptide peaks were detected by absorbance at 210 nm. Fractions of each peak were pooled and freeze-dried. Each sample was dissolved in 0.1 % trifluoroacetic acid and then subjected to HPLC on a Zorbax ODS column (4.6 mm × 150 mm) with a linear gradient from 10 % to 50 % acetonitrile in 0.1 % trifluoroacetic acid for 30 min at a flow rate of 1 ml/min. Amino acid sequence determination was performed with a Shimazu protein sequencer, PPSQ-10.

The amino acid sequences of five peptides and the retention times (in min) of each peptide on each column (Pepkat, Zorbax)

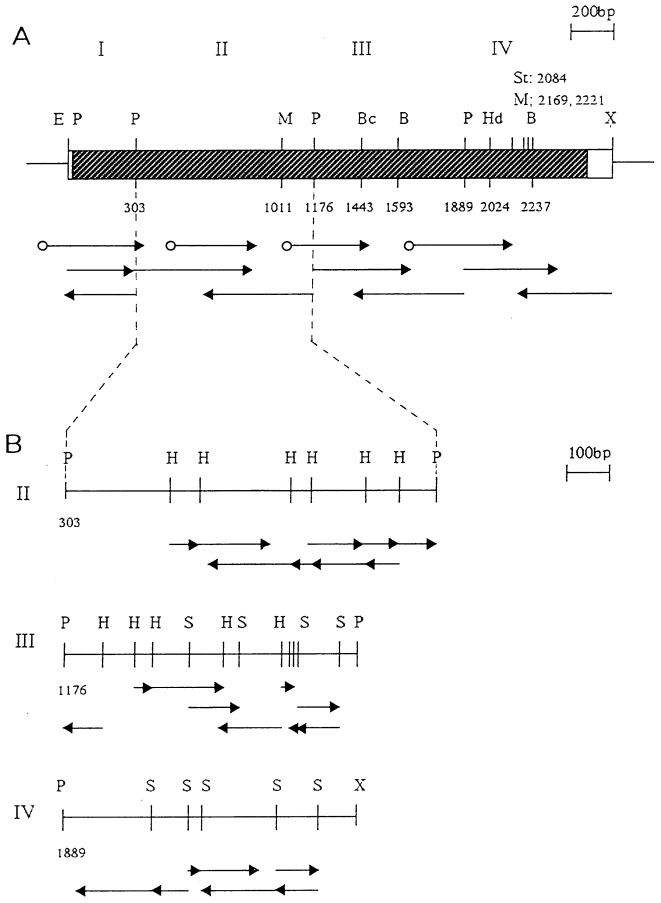

Figure 1 Schematic structure, restriction map and sequencing strategy for DPP III

(**A**) Full-length cDNA. (**B**) II, III and IV regions of cDNA. Symbols and abbreviations: open bar, 5′- and 3′-non-coding regions; hatched bar, protein-coding regions; arrows, direction and extent of each sequence determination; ○, synthesis primers; E, *Eco*RI; P, *Pst*I; X, *Xho*I; M, *Msc*I; Bc, *Bcl*I; B, *Bgl*II; Hd, *Hin*dIII; St, *Stu*I; S, *Sau*3AI; H, *Hae*III. Restriction sites are marked and the nucleotide numbers are shown.

were as follows: peptide 1, LYAHHLSR (14.1, 15.5); peptide 2, LAQDFLDSQNLSAY (2.4, 27.0); peptide 3, SYEFQGNHFQVTR (22.5, 9.6); peptide 4, FWIQDK (23.8, 7.2); peptide 5, SGETWDSK (9.7, 3.6).

RESULTS

Purification of the enzyme from human placenta

The purification was 1775-fold and the yield was 34% with respect to the total activity of the $(NH_4)_2SO_4$ fraction (Table 1). Analysis of column fractions by SDS/PAGE revealed that the major inactive protein contained in the sample was eliminated by a hydroxyapatite column chromatography step (Figure 2A). In this step, if 2-mercaptoethanol was not contained in the buffer, the activity of the eluted DPP III was completely lost even if the enzyme was kept at 4 °C for a few days; activity was not restored by the addition of metal ions and 2-mercaptoethanol. Moreover, contaminating aminopeptidase was thoroughly separated from DPP III, so bestatin was omitted from the assay system in the subsequent steps. SDS/PAGE and Western blot analyses of the placental enzyme and of the recombinant enzyme purified by the same conventional column chromatographies used for the natural

Table 1 Purification of DPP III from human placentas (496 g wet weight)

Step	Total activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
$(NH_4)_2SO_4$ fraction (40–65%)	31.1	3484	0.008	100	1
DEAE-Toyo	27.1	338	0.08	87	10
Hydroxyapatite	13.2	11.4	1.15	42	143
Phenyl-Sepharose	12.9	3.20	4.03	41	503
Sephacryl-S-300	10.8	0.756	14.2	34	1775

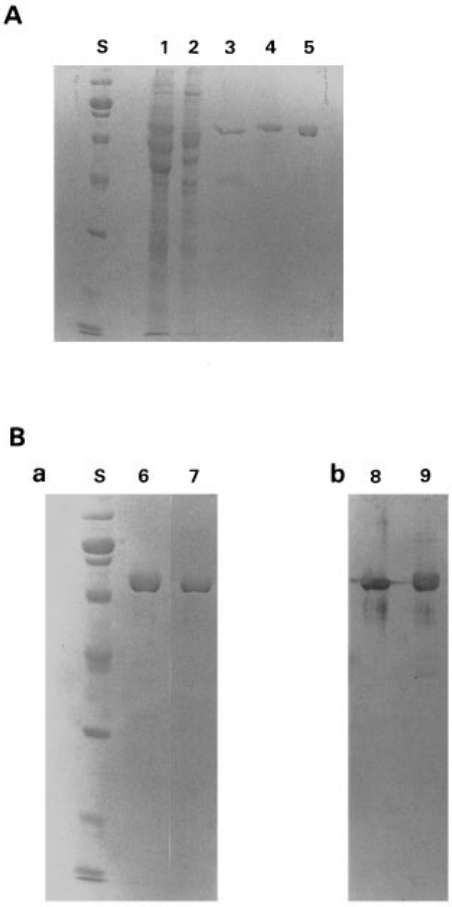

Figure 2 SDS/PAGE analysis of the enzyme fraction during the purification of human placental DPP III (A) and the enzymes purified from human placenta and recombinant *E. coli* cells (B)

Electrophoresis was performed on a 10% (w/v) polyacrylamide gel and stained with Coomassie Blue [**A**, **B**(**a**)]. [**B**(**b**)] Western blot analysis of the purified enzymes. Lanes S, reference proteins; lane 1, fraction after $(NH_4)_2SO_4$ (93 μg); lane 2, fraction after anion exchange (DEAE-Toyo) (26 μg); lane 3, fraction after hydroxyapatite (25 μg); lane 4, fraction after phenyl-Sepharose (10 μg); lane 5, fraction after gel filtration (Sephacryl-S-300) (6 μg); lane 6, recombinant enzyme (8 μg); lane 7, human placenta enzyme (8 μg); lane 8, recombinant enzyme (1 μg); lane 9, human placenta enzyme (1 μg).

enzyme (see the Experimental section) are shown in Figure 2(B). The enzymes were stable in 50% (v/v) glycerol at −20 °C for 1 year without loss of activity.

Table 2 Properties of purified enzymes

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m	Molecular mass (kDa)	Hydrolysis ratio (%) (Lys-Ala/ Arg-Arg)	Zinc content (mol/mol of enzyme)
Placental	Arg-Arg-NA	428 ± 56	83.7	195000	92.7 ± 1.8*	26.0%	0.83
	Lys-Ala-NA	393 ± 45	19.7	50100			
Recombinant	Arg-Arg-NA	371 ± 56	16.6	44700	93 ± 1.5†	11.5%	1.03
	Lys-Ala-NA	73 ± 5	0.962	13100			

* By SDS/PAGE.
† By Sephacryl-S-300 column.

Table 3 Effect of inhibitors on enzyme activity

Data indicate concentration required for 50% inhibition of placental DPP III. NEM, *N*-ethylmaleimide.

Enzyme activity	Potential inhibitor	Inhibitor concentration for 50% inhibition (μM)
Metallopeptidase	*o*-Phenanthroline	240 ± 16
	EDTA	298 ± 11
Cysteine peptidase	PCMPS	3.30 ± 0.24
	NEM	6330 ± 640
Serine peptidase	DCI	4.54 ± 0.74
	PMSF	2170 ± 470

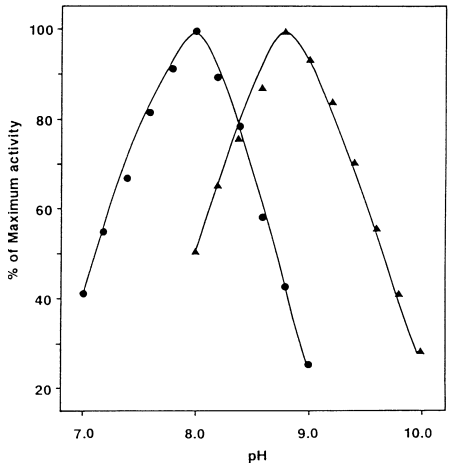

Figure 3 Effect of pH on activities of purified enzymes against Arg-Arg-NA

Symbols: ▲, placental enzyme; ●, recombinant enzyme.

Properties of the purified enzymes

The enzymes hydrolysed the substrate Arg-Arg-NA most rapidly; the activity with Lys-Ala-NA was only 26.0% (placenta) and 11.5% (recombinant) of that observed with Arg-Arg-NA at physiological pH 7.4 (Table 2). The other substrates used were not hydrolysed at all. K_m values (for Arg-Arg-NA) of the enzymes under the standard assay condition were of almost the same order (300–400 μM)], although those (for the same synthetic substrate) of DPP III reported previously covered a broad range, i.e. 6.0 mM to 7.7 μM [4,5]. Catalytic efficiency (k_{cat}/K_m) of the placental enzyme was about 4-fold that of the recombinant enzyme for both substrates. Inhibitor concentrations required for 50% inhibition of the control activity are shown in Table 3. The effects of inhibitors on both enzymes were not significantly different. The IC_{50} of metal chelators such as *o*-phenanthroline and EDTA was of the order of 100 μM. The non-chelating analogues 1,7-phenanthroline and 4,7-phenanthroline did not inhibit at all (results not shown). PCMPS, an organomercurial compound, was effective (1 μM) but *N*-ethylmaleimide, a general reagent for thiol groups, was a poor inhibitor (1 mM). *trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), a specific inhibitor of papain-family enzymes (cysteine peptidase) [17], did not inhibit at all even at a final concentration of 1 mM (results not shown). Serine peptidase inhibitors such as PMSF and 1 mM DFP (results for latter not shown) did not inhibit the enzyme effectively. Of them, DCI was the most effective (1 μM). As inhibition by *o*-phenanthroline was genuinely due to metal chelation, we analysed the zinc content of the enzymes; they contained 0.727, 0.879, 0.891 (human placenta) and 0.970, 1.05, 1.08 (recombinant) mol of zinc per mol of protein. The mean value is shown in Table 2.

The pH dependence of the enzyme activities is shown in Figure 3. The optimum pH of the human placental enzyme with Arg-Arg-NA used as substrate was 8.8, consistent with previously reported values for DPP III forms [1–6]. On the other hand, that of the recombinant enzyme was 8.0, the same as the value for the rat skin and yeast enzymes [18,19]. DPP III purified from rat liver by the same purification methods as used for the human placental enzyme also had an optimum pH at 8.0 (results not shown). The difference in the pH optimum (towards artificial substrate) of the enzymes from both species depends on the species and organ of origin, because it is known that it (for native substrate, such as enkephalin) is shifted to the same neutral range (pH 7.0–7.5) [20,21].

Zinc binding study

In Figure 4 the activity response of DPP III is plotted against the logarithm of the free Zn^{2+} ion concentrations computed from the composition of the 2,6-PA/Zn^{2+} buffer. The relationship between the logarithm of free Zn^{2+} ions and the activity of DPP III gave a sigmoidal curve. At 10 fM Zn^{2+}, DPP III was almost inactive. Most of the activity was reconstituted by increasing the concentration of free Zn^{2+} to 3 pM.

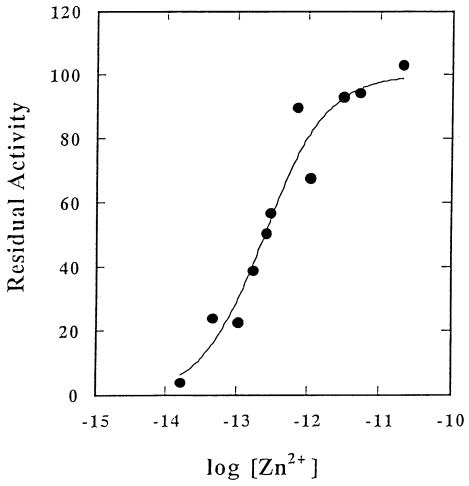

Figure 4 Relationship between residual enzyme activity and log[Zn^{2+}]

The assays were conducted in 100 μl of 50 mM phosphate buffer, pH 7.4, containing 900 μM–25 mM 2,6-PA and 100 μM $ZnSO_4$. After equilibration between apo-DPP III and 2,6-PA for the Zn^{2+} ions (60 min incubation at 25 °C), reactions for the enzyme activity were performed with the standard enzyme assay described in the Experimental section.

By using eqn. (7), the zinc dissociation constant of apo-DPP III was calculated from the relationship between the free Zn^{2+} ions and the enzyme activity. In Figure 4 the theoretical curve obtained from eqn. (7), in which apo-DPP III reacts with only one Zn^{2+} ion, is consistent with the results obtained for enzyme activity. This behaviour clearly indicates that DPP III has only one Zn^{2+} ion at the active site. The zinc dissociation constant (K_d) calculated from eqn. (7) was 250 ± 50 fM at pH 7.4 (50 mM phosphate buffer). This low dissociation constant of the Zn^{2+} ions in the enzyme clearly shows that DPP III is a metalloenzyme. The value of the zinc dissociation constant in DPP III is almost consistent with that of bovine pancreas carboxypeptidase A (K_d 30 pM at pH 8.0 [22]), in which the co-ordination residues are His, His and Glu, and almost consistent with that of bovine carbonic anhydrase (K_d 3 pM at pH 7.4 [23]), in which the co-ordination residues are His, His and His. However, the dissociation constant of the Zn^{2+} ions for yeast aminopeptidase I [24] was 10^4-fold larger than that for DPP III.

Molecular cloning of DPP III

A rat liver cDNA library was screened immunologically; five positive clones were isolated. To express the protein in *E. coli* we constructed the pBluescript phagemids from these positive clones. Three of the five clones had a high Arg-Arg-NA-hydrolysing activity, so we expected them to contain a full-length cDNA. The molecular masses of the insert cDNA and the nucleotide sequences of the 5′ regions of the insert cDNA of these three clones were the same; therefore we concluded that the clones were identical.

The nucleotide sequence and the deduced amino acid sequence of the cDNA of one of the clones are shown in Figure 5. DNA sequence analysis revealed that this cDNA contained a 3′ poly(A) tail preceded by a poly(A) signal 13 nt upstream.

In the open reading frame, five potential translational start sites were identified in the 5′ nucleotide region, and we numbered the A of the first ATG codon as 1. The fourth methionine residue at nt 532 corresponds to Kozak's rule [25] so if the open reading frame started at this position, the cDNA would encode a 642-residue polypeptide of theoretical molecular mass 72044 Da. On the other hand, the longest open reading frame of 2481 bp would encode an 827-residue polypeptide of theoretical molecular mass of 92790 Da. The molecular masses of the purified enzymes from human placenta and recombinant *E. coli* cells were equal and estimated to be 93 kDa by gel filtration and 92.7 kDa by SDS/PAGE (Table 2). Furthermore one of the tryptic peptides of the recombinant enzyme (peptide 1) was found in the N-terminal domain of the longest open reading frame. These two pieces of evidence suggest that translation might be initiated at the first AUG.

```
                                                                ctgcagcagggctc  -14
ATGGCAGATACCCAGTACATCCTGCCCAATGACATCGGTGTGTCTAGCCTGGACTGCCGGGAGGCATTCCGCCTGCTGTCACCCACAGAG    90
M A D T Q Y I L P N D I G V S S L D C R E A F R L L S P T E                                    30

CGCCTCTATGCCCATCACCTGTCTAGAGCCGCTTGGTATGGAGGCCTGGCTGTGCTGCTCCAGACATCTCCTGAAGCCCCCTACATCTAT  180
R L Y A H L S R A A W Y G G L A V L L Q T S P E A P Y I Y                                    60

GCCCTGCTTAGCCGCCTCTTCCGTGCCCAGGACCCTGACCAGCTACGCCAACATGCCCTGGCTGAGGGCCTGACTGAAGAAGAGTATCAG  270
A L L S R L F R A Q D P D Q L R Q H A L A E G L T E E E Y Q                                    90

GCATTCCTGGTCTACGCTGCAGGGGTCTACTCCAACATGGGCAACTATAAGTCCTTCGGTGACACCAAGTTTGTCCCTAACCTGCCCAAG  360
A F L V Y A A G V Y S N M G N Y K S F G D T K F V P N L P K                                   120

GAAAAGCTGGAGCGTGTGATCCTTGGGAGTAAGGCTGCCCAACAGCATCCAGAAGAAGTCAGGAGCCTTTGGCAGACCTGTGGGGAGCTT  450
E K L E R V I L G S K A A Q Q H P E E V R S L W Q T C G E L                                   150

ATGTTCTCCCTGGAACCAAGGCTTCGACATCTTGGGCTGGGGAAGGAGGGAGTCACCACCTATTTCTCTGGGGATTGTGCCATGGAAGAT  540
M F S L E P R L R H L G L G K E G V T T Y F S G D C A M E D                                   180

GCCAAGCTGGCCCAGGACTTCCTGGACTCTCAGAACCTCAGTGCATACAACACCCGGCTCTTCAAGGTGGTCGGCCAGGAAGGGAAGTAC  630
A K L A Q D F L D S Q N L S A Y N T R L F K V V G Q E G K Y                                   210

CACTATGAGGTGCGACTGGCTTCAGTACTCAACACAGAACCTGCTCTCGACTCTGAGTTGACTTCCAAGCTGAAGAGCTACGAGTTCCAG  720
H Y E V R L A S V L N T E P A L D S E L T S K L K S Y E F Q                                   240

GGGAATCATTTCCAGGTCACCCGTGGGGACTATGCCCCCATCCTCCAGAAGGTGGTAGAACACTTGGAGAAAGCTAAGGCTTATGCAGCC  810
G N H F Q V T R G D Y A P I L Q K V V E H L E K A K A Y A A                                   270

AATAGCCACCAGGAGCAAATGCTGGCCCAGTATGTGGAGAGCTTCACCCAGGGTTCCATTGAGGCCCATAAGAGGGGCTCCCGCTTCTGG  900
N S H Q E Q M L A Q Y V E S F T Q G S I E A H K R G S R F W                                   300

ATTCAGGACAAAGGTCCCATTGTAGAGAGTTACATTGGCTTCATTGAGAGCTACCGTGACCCCTTTGGCTCCCGAGGAGAATTTGAAGGC  990
I Q D K G P I V E S Y I G F I E S Y R D P F G S R G E F E G                                   330

TTCGTGGCCATGGTAAACAAAGACATGAGTGCAAAGTTCGAGCGGCTGGTTGCAAGTGCTGAGCAGCTGCTGAAGGAGCTGCCTTGGCCT 1080
F V A M V N K D M S A K F E R L V A S A E Q L L K E L P W P                                   360

CCTGCCTTTGAGAAGGACAAGTTCCTCACCCCTGACTTCACTTCCCTGGATGTCCTTACCTTTGCCGGCTCTGGCATCCCTGCAGGCATC 1170
P A F E K D K F L T P D F T S L D V L T F A G S G I P A G I                                   390

AACATCCCCAACTATGATGACCTGAGACAGACAGAGGGCTTTAAGAATGTGTCTCTGGGGAATGTGCTGGCTGTGGCCTATGCCACAAAG 1260
N I P N Y D D L R Q T E G F K N V S L G N V L A V A Y A T K                                   420

CGGGAGAAACTCACCTTCATGGAGGAAGAAGACAAGGACCTGTACATCCGCTGGAAGGGGCCGTCCTTTGATGTGCAGGTGGGGTTACAT 1350
R E K L T F M E E E D K D L Y I R W K G P S F D V Q V G L H                                   450

GAGCTGTTGGGCCATGGCAGCGGCAAGCTCTTTGTACAGGATGAGAAAGGTGCTTTCAACTTTGACCAGGAGACAGTGATCAACCCAGAG 1440
E L L G H G S G K L F V Q D E K G A F N F D Q E T V I N P E                                   480

ACTGGGGAGCAGATCCAGAGCTGGTACCGGAGTGGAGAGACATGGGATAGCAAATTTAGCACCATTGCCTCCAGCTACGAAGAGTGCCGG 1530
T G E Q I Q S W Y R S G E T W D S K F S T I A S S Y E E C R                                   510

GCTGAGAGCGTGGGCCTCTACCTCTGTCTCAACCCCCAAGTACTTCAGATCTTTGGCTTTGAGGGAACTGACGCAGAAGATGTCATCTAT 1620
A E S V G L Y L C L N P Q V L Q I F G F E G T D A E D V I Y                                   540

GTGAACTGGCTCAACATGGTCCGGGCTGGGCTGTTGGCTTTGGAGTTCTACACCCCAGAGACGGCCAACTGGCGACAGGCCCACATGCAG 1710
V N W L N M V R A G L L A L E F Y T P E T A N W R Q A H M Q                                   570

GCCCGGTTTGTGATCCTGAGGGTTTTGCTGGAAGCTGGCGAAGGACTCGTTACCGTCACCCCCACCACAGGCTCTGATGGGCGCCCAGAT 1800
A R F V I L R V L L E A G E G L V T V T P T T G S D G R P D                                   600

GCTCGTGTCCACCTGGACCGCAGCAAGATCCGGTCAGTGGGCAAACCTGCTTTGGAGCGGTTCCTCCGGAGACTGCAGGTGCTGAAGTCC 1890
A R V H L D R S K I R S V G K P A L E R F L R R L Q V L K S                                   630

ACAGGGGATGTGGTTGCAGGGCGCGCCCTGTACGAGGGCTATGCGGCGGTCACTGACGCACCCCCAGAGTGCTTCCTCACTCTAAGGGAC 1980
T G D V V A G R A L Y E G Y A A V T D A P P E C F L T L R D                                   660

ACGGTATTGCTGCGCAAGGAATCCCGGAAGCTTATTGTCCAGCCCAACACTCGCCTGGAAGGCTCAGAAGTGCAACTTGTAGAGTATGAG 2070
T V L L R K E S R K L I V Q P N T R L E G S E V Q L V E Y E                                   690

GCCTCAGCGGCTGGTCTCATCCGATCCTTCTGTGAACGGTTTCCGGAGGATGGGCCTGAGTTGGAGGAGGTTCTTACCCAGCTGGCCACA 2160
A S A A G L I R S F C E R F P E D G P E L E E V L T Q L A T                                   720

GCAGATGCTCAATTCTGAGAGGGATCAGGTCCAGGAGGCCCCATCTGGCCAGGCTTTGAGAAGATCTGTGTGGCATCTCCCCTTTCATCAA 2250
A D A Q F W R D Q V Q E A P S G Q A L R R S V W H L P F H Q                                   750

ACCAGGGCTGTCTGTGGCCCTGAATTGTGTTTAGGAGGTGGGGGAAGGGCAGGAGCTCGGACTTTGGTGCTACCTCAGCTGAGGGTGGTG 2340
T R A V C G P E L C L G G G G R A G A R T L V L P Q L R V V                                   780

ATGCTAACCCCTTCCATTTGTCAGCACTTTCCAGTTTACCATTATGTCCTCATTTCCTGGATCTGCACTGCCATCTGTGGTGGGAGCTCA 2430
M L T P S I C Q H F P V Y H Y V L I S W I C T A I C G G S S                                   810

ACAGGGTGCCTAGTCTGGTTTTCCAAATGGGAAGGTGGCAGTTCTGAGAAGTAActgttctagatccagcaggtggcatgtgacagagcc 2520
T G C L V W F S K W E G G S S E K                                                             827

agggctcaaagcattctcaccaaatccagtgctcttcgtgtattacctttatagccagaaaaataaatatgacaaccaccaaaaaaaaa 2610
aaaaaaaaa                                                                                    2619
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Figure 5 Nucleotide sequence of the cDNA and the deduced primary structure of rat liver DPP III

Nucleotide residues are numbered from 5′ to 3′, with the first residue of the ATG codon encoding the putative initiating methionine. The deduced amino acid sequence is displayed below the nucleotide sequence as a single-letter code starting from methionine. The sequences matching those of peptide fragments identified after tryptic digestion of the recombinant DPP III are underlined. The putative zinc-binding motif of metallopeptidases is boxed. The putative polyadenylation signal is identified by a double line.

DISCUSSION

Arg-Arg-NA, along with other dipeptide-NAs, was used to characterize the spectrum of peptidases in the anterior pituitary gland; dipeptidyl arylamidase III was discovered to be one of the DPPs cleaving Arg-Arg-NA at pH 9.0. When naturally occurring

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DPPIII  184 AQDFLDSQNLSAYNTRLFKVVGQEGKYHYEVRLASVLNTE  223
S.cer.    1 MALLKEQLFAELAILPENTRINKV*ENSFQIWV**E-*VK   39

DPPIII  224 PALDSELTSKLKSYEFQGNHFQVTRGDYAPILQKVVEHLE  263
S.cer.   40 NQITETYP*GQITLSNAVTKVEFIF**HSREMRL*ASY*K   79

DPPIII  264 KAKAYAANSHQEQMLAQYVESFTQGSIEAHKRGSRFWIQD  303
S.cer    80 E*QKF***DT*KA**QE*INHFVTGSSQ***EAQKL*VK*  119

DPPIII  304 KGPIVESYIGFIESYRDPFGSRGEFEGFVAMVNKDMSAKF  343
S.cer   120 IS*VI*TN*****T**E*S*II****SL**IQ**ERT***  159

DPPIII  344 ERLVASAEQLLKELPWPPAFEKDKFLTPDFTSLDVLTFAG  383
S.cer   160 SS**NN**EFISL***SKDY**PI*NP******E**TFT*  199

DPPIII  384 SGIPAGINIPNYDDLRQTEGFKNVSLGNVL-AVAYATKRE  422
S.cer   200 **************V*LKI*********I*S*A*KSSSKH  239

DPPIII  423 KLTFMEEEDKDLYIRWKGPSFDVQVGLHELLGHGSGKLFV  462
S.cer   240 PPS*ISQ**RPIFEKYQSD**E****I==========LT  279

DPPIII  463 QDEKGAFNFDQETVINPETGEQIQSWYRSGETWDSKFSTI  502
S.cer   280 EFTD*-****K=NPPLGLD*KPVSTY*KV****G***GQL  318

DPPIII  503 ASSYEECRAESVGLYLCLNPQVLQIFGFEGTDAED-VIYV  541
S.cer   319 *GOF******VIAMF*LT*KKI*D****HDVESQ*K***A  358

DPPIII  542 NWLNMVRAGLLALEFYTPETANWRQAHMQARFVILRVLLE  581
S.cer   359 GY*Q*A********YWN*K*GK*G*P******S*MKTFMK  398

DPPIII  582 AGEGLVTVTPTTGSDGRPDARVHLDRSKIRSVGKPALERF  621
S.cer   399 HSTDKNFLKLEMN*-TND*FAIK**K*L*KTA*HECVKDY  437

DPPIII  622 LRRLQVLKSTGDVVAGRALYEGYAAVTDAPPECFLTLRDT  661
S.cer   438 *KH*H*Y*CS***EQ*SKYFIDRST**---*D-LAS***I  873

DPPIII  662 VLLRKESRKLIVQPNTRLEGSE-VQLVEYEASAAGLIRSF  700
S.cer   874 **SKRLP*RQFI*S*SYIDDNNK*T*K**DETPQ*MLQ**  513

DPPIII  701 CERFP  705
S.cer   514 LD*EL  518
```

Figure 6 Alignment of the predicted rat DPP III protein sequence with that of the hypothetical *S. cerevisiae* (*S. cer*) protein 01232

Residues identical with those of DPP III are indicated by asterisks in the yeast protein sequence. For optimal alignment, several gaps were inserted into the *S. cer* sequence. The putative zinc-binding motif of metallopeptidases is indicated by a double underline.

peptide substrates such as angiotensin and enkephalin were used, the optimum pH was in the physiological range [4,21]. We used the buffer reported by Lee and Snyder for our enzyme assay. Although DPP III is typically a cytosolic enzyme, a similar enzyme has been purified from a brain membrane fraction [26–28]. The enzyme, designated as enkephalinase B (enkephalin-degrading dipeptidyl aminopeptidase), was inhibited by DFP as well as by metal-chelating agents [27]. However, on the basis of ingenious experiments with special inhibitors, enkephalinase B was reported to be a metalloenzyme [20,29]. The relationship between DPP III and enkephalinase B is not known.

We determined the zinc content of the placental enzyme by an atomic absorption experiment and showed the Zn^{2+} ion to reside in the catalytic domain by a zinc-binding study. Moreover, we described the isolation and the sequence determination of a cDNA encoding rat DPP III. The deduced amino acid sequence contained all peptide sequences that were obtained by enzymic fragmentation of the purified recombinant enzyme and sequence determination of these fragments.

Interestingly, the cDNA predicted a protein that contains a sequence (HE*LL*GHX_{18}E) similar to the HE*XX*H consensus sequence of metallopeptidases. Rawlings and Barrett [30] divided the 30 families of the metallopeptidases into five groups. Family M1 of clan MA contains the HE*XX*H motif with an additional glutamic residue 18 residues apart from it; this motif constitutes the active site of metallopeptidases such as Ap-A, N and B [31]. The other families of clan MA contain the HE*XX*H motif with an additional glutamic residue 19 residues apart, as in thermolysin, peptidyl peptidase A and mycolysin. The zinc-binding site of the former enzymes is at (H^1– H^5–E^{24}) of a 24-residue domain and that of the latter enzymes is at (H^1–H^5–E^{25}) [30]. The region around the sequence of the HELLGH motif in DPP III resembled that of the metallopeptidases when a space was inserted to correspond to the additional residue (L) in the motif. In DPP III, Glu-2 was similarly a catalytic amino acid as in metalloenzymes belonging to clan MA, as judged from the results of a site-directed mutagenesis study (results not shown). Although we suppose that the metal-binding site in DPP III lies in a similar sequence domain (H^1–H^6–E^{25}), it should establish whether DPP III is a new type of metalloenzyme.

A search of the NBRF–PIR protein sequence database with the primary structure of DPP III showed significant similarity between it and the hypothetical yeast protein 01232 of *Saccharomyces cerevisiae* (accession no. S59292), i.e. 40.4% identity over 503 residues (Figure 6). Although the physiological function of the yeast protein is not known, the sequence of HELLGHX_{18}E is conserved in its deduced amino acid sequence (Figure 6). In contrast, DPP III has been found in *S. cerevisiae* by other researchers [19]. It is inhibited by metal chelators (IC_{50} 10 μM) and stimulated by the addition of Co^{2+}, as reported for other metalloenzymes [32,33]. The hypothetical protein possibly has the activity of DPP III.

Dipeptides released by the action of DPPs I–IV are hydrolysed to single amino acids in the terminal stage of catabolism. With respect to pH dependence and substrate specificities, DPP III in particular supplies most of the dipeptides at physiological pH.

Although DPP III is involved in the process of inactivation of angiotensin II [34], other physiological functions of DPP III (neutral DPP) remain to be elucidated in further experiments on the localization of the enzyme by immunohistochemistry.

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