Tripeptidyl peptidase II in haemolysates and liver homogenates of various species

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Rabbit antibodies against purified tripeptidyl peptidase II (TPP II) of human erythrocytes have been prepared and used in immunoblot analysis. Antibodies affinity-purified against the denatured 135000- M_r subunit of the human peptidase were shown to cross-react with purified rat liver TPP II, as well as a polypeptide of M_r 135000 in haemolysates and liver homogenates from several other species. TPP II activity in haemolysates from monkey, hog, horse and rabbit corresponded to the levels found in human erythrocytes, whereas this activity was absent or low in erythrocytes from calf and hen. On immunoblot analysis of the calf haemolysates, the 135000- M_r polypeptide could not be detected. In contrast, analysis of liver homogenates from these species revealed both TPP activity and immunoreactive polypeptides. Immunoreactive polypeptides with M_r about 105000 and 84000 of variable occurrence were also detected. In addition, extracts of *Escherichia coli* showed no TPP II activity under the conditions of the standard assay, although polypeptides of M_r 135000 and 40000 were revealed on immunoblot analysis of this species.

INTRODUCTION

Tripeptidyl peptidase II (TPP II), originally detected in the extralysosomal fraction of rat liver homogenates (Bålów *et al.*, 1983), has recently been extensively purified from this source and from human erythrocytes (Bålów *et al.*, 1986). The peptidase from both sources showed several features in common, e.g., chromatographic behaviour, a native M_r above 10^6 , a subunit M_r of 135000, similar specific activity and a neutral pH optimum. The enzyme releases tripeptides with little apparent similarity from a series of peptide substrates (Bålów *et al.*, 1983, 1986). This may reflect a comparatively low substrate specificity of TPP II, suggesting a general function in the extralysosomal proteolysis.

The similarities between the peptidase of human erythrocytes and rat liver suggest common structural features, and a cross-reaction of polyclonal antibodies against the denatured subunit of the erythrocyte enzyme with the liver enzyme could be expected. As shown in the present work, these antibodies recognized the liver enzyme, and the investigation was extended to a comparison of the occurrence of TPP II activity and immunoreactive 135000- M_r subunits in various species. This screening revealed unexpected differences between species. The implications of these results are discussed.

EXPERIMENTAL

Reagents

Freund's adjuvant (complete and incomplete) was obtained from DIFCO Laboratories. The nitrocellulose sheets (0.2 μ m pore size) came from Schleicher and Schüll. M_r standards for electrophoresis, affinity-purified goat anti-rabbit IgG (heavy + light)–horseradish peroxid-

ase conjugate and 1-chloro- β -naphthol were purchased from Bio-Rad. Protein A–Sepharose CL-4B, AH-Sepharose 4B, CNBr-activated Sepharose 4B, Sephadex G-25, and QAE-Sephadex A-25 were obtained from Pharmacia Fine Chemicals. The 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride came from Chemalog. Lysozyme, microcrystalline cellulose (average particle size 50 μ m), and α -cellulose were procured from Sigma. BSA (fraction V) was obtained from Boehringer Mannheim G.m.b.H. The human IgG came from Kabi Vitrum. The poly(ethylene glycol) 6000 ('puriss' grade) was bought from Kebo. All other chemicals used were of analytical grade.

Purified TPP I from hog ovaries was kindly provided by Professor Ken McDonald, Medical University of South Carolina, Charleston, SC, U.S.A. Human venous blood was obtained from healthy volunteers. Small portions of macroscopically normal human liver tissue were excised during liver surgery at the University Hospital of Uppsala. Rabbit liver, and blood from monkey and rabbit, were taken from animals kept at the local laboratory animal house. Blood and liver from hog and calf were supplied by the local slaughterhouse. Hen blood and liver were taken from birds kept at the Swedish University of Agricultural Sciences. Equine blood samples were obtained by courtesy of Dr. Nils Juntti, The National Veterinary Institute, Uppsala, Sweden.

Preparation of haemolysates and liver homogenates

Blood samples (collected in the presence of EDTA or EGTA) were centrifuged and the plasma was discarded. The subsequent steps were performed at 4 °C. The remaining blood cells were washed twice in 0.9% NaCl, and 1 vol. of packed cells was lysed with 8 vol. of

Abbreviations used: anti-135 kDa, antibodies affinity-purified against denatured 135000- M_r subunit of TPP II (tripeptidyl peptidase II); anti-(TPP II), antibodies affinity-purified against native TPP II; BSA, bovine serum albumin; DTT, dithiothreitol; PEG, poly(ethylene glycol) 6000; AH-, aminohexyl; QAE-, quaternary aminoethyl; s.c., subcutaneously; i.p., intraperitoneally.

0.01 M-potassium phosphate, pH 7.5, containing 2 mM-EGTA, and 1 mM-DTT. The haemolysate was centrifuged at 10000 *g* for 15 min and the supernatant was frozen in liquid N₂ and stored at -70 °C until use. Hen blood cells used in immunoblot analysis were washed as described above and lysed with 8 vol. of 10 mM-potassium phosphate, pH 7.5, containing 5 mM-MgCl₂, 2 mM-EDTA, 2 mM-EGTA and 1 mM-DTT. The haemolysate was centrifuged and the supernatant frozen and stored as described above. Samples used in immunoblot analysis were concentrated to one-third of the initial volume in an Amicon CS 15 ultra-filtration cell and incubated with SDS sample buffer as described below for SDS/polyacrylamide-gel electrophoresis.

Liver homogenates were prepared as described by Bålów *et al.* (1983). The supernatants from centrifugation at 25000 *g* for 15 min were frozen in liquid N₂ and stored at -70 °C until analysis. Samples used in immunoblot analysis were incubated with SDS sample buffer as described below.

Assay of TPP II activity

The enzyme activity was measured by the standard assay previously described (Bålów *et al.*, 1983). One unit of TPP II will hydrolyse 1.0 nmol of Arg-Arg-Ala-Ser([³²P]P)-Val-Ala/min at pH 6.5 and 30 °C. The ³²P-labelled product of the reaction, Ser([³²P]P)-Val-Ala, was collected from the reaction mixture by the binding of QAE-Sephadex and measured as Čerenkov radiation. The cleavage products were also analysed by the electrophoretic method (Bålów *et al.*, 1983).

Production of rabbit anti-(human TPP II) and isolation of IgG

Rabbits were injected s.c. with 50 mM-potassium phosphate, pH 7.5, in 30% (w/v) glycerol emulsified in 1 vol. of Freund's complete adjuvant, the total volume of emulsion being 0.3 ml/animal. The human erythrocyte peptidase (0.25 mg/0.3 ml of emulsion), purified as described (Bålów *et al.*, 1986) and mixed with Freund's incomplete adjuvant, was injected 2 weeks later, followed by three booster injections at 4-week intervals with the same amount of antigen in Freund's incomplete adjuvant. The animals were bled and killed 1 month after the fourth booster injection. The IgG fraction of the antisera was isolated by chromatography on protein A-Sepharose as described by Hjelm *et al.* (1972) and stored at -25 °C.

Isolation of the 135000-*M_r* subunit of TPP II

Preparative SDS/8% (w/v)-polyacrylamide-slab-gel electrophoresis was performed in the discontinuous system of Laemmli (1970) as described by O'Farrell (1975), except that the thickness of gels was 3 mm. The gel was loaded with 3.8 mg of purified peptidase and the electrophoresis was run at 16 °C for 42 h. The area corresponding to the position of the 135000-*M_r* subunit was cut into 3 mm × 3 mm × 3 mm pieces and equilibrated for 40 min in 6 mM-Tris/HCl, pH 8.3, containing 48 mM-glycine and 10 mM-DTT. The polypeptide was electrophoretically eluted into the same buffer for 19 h at 400 V as described by Rubin *et al.* (1986), to give a 135000-*M_r* polypeptide yield of 1 mg.

Affinity purification of rabbit anti-(human TPP II) with immobilized peptidase 135000-*M_r* subunit

All steps of the procedure described below were performed at 4 °C. A 1 mg portion of purified 135000-*M_r* polypeptide, dialysed overnight against redistilled water, was linked to 0.3 g of AH-Sepharose in the presence of 6 mM-1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride as described by the manufacturer. A 1 ml column was prepared and equilibrated with PBS (20 mM-sodium phosphate/0.15 M-NaCl, pH 7.5) containing 0.2% (v/v) Tween 80. The IgG fraction of rabbit anti-(human TPP II) (9.5 mg of protein in 3 ml), prepared as described above, was drained into the affinity column at a rate of 4 ml/h. After washing with 10 ml of the equilibration buffer the column was eluted with 4 ml of 3.5 M-MgCl₂ and subsequently eluted with 2 ml of equilibration buffer. The eluate was immediately desalted on a Sephadex G-25 column equilibrated with PBS. The protein content of the desalted samples ranged between 0.04 and 0.07 mg/ml. The material was stored at 4 °C in the presence of 15 mM-NaN₃.

Affinity purification of rabbit anti-human IgG with immobilized native peptidase

All the following procedures were carried out at 4 °C. A 0.4 mg portion of native enzyme, dialysed overnight against coupling buffer (0.1 M-NaHCO₃, pH 8.4, in 0.5 M-NaCl) was coupled to 0.8 ml of swollen CNBr-activated Sepharose 4B as recommended by the manufacturer. A 0.8 ml column was prepared, equilibrated, loaded with 2 ml of IgG preparation and eluted as described above for the affinity chromatography against immobilized 135000-*M_r* polypeptide. After desalting on a Sephadex G-25 column the material (0.04–0.08 mg of protein/ml) was stored at 4 °C in the presence of 15 mM-NaN₃.

Preparation of lysates of rat reticulocytes

Newborn Hooded Lister rats (1–6 days old, 6–10 g body wt.) were anaesthetized by injection (i.p.) of sodium pentobarbital (2.5 mg/animal) and the blood was collected by heart puncture. The following procedures were carried out at 4 °C. In order to remove white blood cells, 1 ml of blood was applied to a mixture of microcrystalline cellulose and α-cellulose (1:1, w/w) equilibrated in 0.9% NaCl and packed in a column (1.4 cm × 4 cm) (Beutler & West, 1976). The eluted blood cells were collected by centrifugation and lysed by the addition of 1.6 vol. of redistilled water. The lysate was dialysed for 2 h against 500 vol. of 8 mM-KCl, 4 mM-MgCl₂ and 1 mM-DTT, frozen in liquid N₂ and stored at -70 °C until tested.

Preparation of *Escherichia coli* extracts

E. coli, strain HB101 (Boyer & Roulland-Dussoix, 1969) was grown at 37 °C with aeration in 50 ml of LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 0.5% NaCl) and collected by centrifugation at an *A*₆₀₀ of 2.5. The cells (8 × 10¹⁰) were frozen at -70 °C for 1 h, thawed and lysed by a modification of the procedure of Birnboim & Doly (1979). The cells, suspended in 1.5 ml of 50 mM-glucose/10 mM-EDTA/25 mM-Tris/HCl, pH 8.0, were treated with lysozyme (100 μg/ml) for 30 min at 4 °C, and DTT was added to a final

concentration of 2 mM. The lysate was centrifuged at 40000 *g* for 30 min, and the supernatant was frozen in liquid N₂ and stored at -70 °C until analysis.

Polyacrylamide-gel electrophoresis in SDS

Analytical polyacrylamide-gel electrophoresis was performed as described for the preparative slab gels above, except that the gel thickness was 1 mm. The acrylamide concentration of the running gel was 8% (w/v). Before application the samples were rendered 2.3% (w/v) in SDS, 5% (v/v) in β -mercaptoethanol and 10% (w/v) in glycerol, and heated in a boiling-water bath for 3 min. After electrophoresis, except when proteins were to be transferred to nitrocellulose, the proteins were fixed for 1 h with 10% (w/v) trichloroacetic acid and stained as described by Blobel & Dobberstein (1975).

Immunoblot analysis

Polypeptides separated on SDS/polyacrylamide gels were electrophoretically transferred to nitrocellulose for 24 h in 0.02 M-Tris/0.15 M-glycine (pH 8.4)/10% (v/v) methanol/0.01% (w/v) SDS, at 4.5 V/cm. Before transfer the gels and the nitrocellulose sheets were equilibrated in transfer buffer for 20 min at room temperature. After transfer, the nitrocellulose filter was incubated for 3 h at room temperature with 3% (w/v) gelatin in PBS and thereafter for 12–16 h at 4 °C with affinity-purified antibodies (4–6 μ g in 5 ml) diluted in PBS containing 1% (w/v) BSA and 3% (w/v) PEG. The filter was washed for 30 min with three changes of washing buffer [PBS (pH 7.5)/0.5% (v/v) Tween 20/3% PEG]. Horseradish-peroxidase-conjugated goat anti-rabbit IgG (2.5 μ g in 5 ml), diluted in the same buffer as used for the primary antibodies, was added and the nitrocellulose was incubated for an additional 2 h. The filter was washed with four changes of washing buffer and the antigen was detected by incubation for 3 h with 1-chloro- β -naphthol (0.6 mg/ml) in PBS (pH 7.5)/3% PEG in the presence of 0.018% (v/v) H₂O₂. The reaction was terminated by addition of 15 mM-NaN₃ in PBS.

Analytical methods

Protein was measured with the method of Bradford (1976) as described by Read & Northcote (1981), either BSA or human IgG being used as standards.

RESULTS

TPP II activity of haemolysates of various species

As can be seen in Table 1, the peptidase activity in haemolysates of monkey, hog, horse and rabbit is comparable with the activity found in human and rat haemolysates, i.e. about 50 units/ml of packed blood cells (Bålów *et al.*, 1986). The TPP II activity in these crude tissue extracts was measured with both the standard assay and the electrophoretic method previously described (Bålów *et al.*, 1983). The latter method discriminates between the phosphopeptides Ala-Ser(P)-Val-Ala and Ser(P)-Val-Ala, both of which may be formed from the standard substrate Arg-Arg-Ala-Ser(P)-Val-Ala by crude tissues extracts and bound to the QAE-Sephadex of the standard assay. By the electrophoretic method it was confirmed that Ser(P)-Val-Ala was the predominant cleavage product on incubation with lysates of human, monkey, hog, horse and rabbit erythrocytes. In addition, the low levels of peptidase

Table 1. TPP activity in haemolysates from different species

Haemolysates were prepared as described in the Experimental section. Peptidase activity was measured by the standard assay. Activity values refer to 1 ml of packed blood cells.

Species	Enzyme activity (units) [mean $\pm$ S.D. (n)]
Human	50 $\pm$ 11 (6)
Monkey	39 $\pm$ 2 (3)
Hog	49 $\pm$ 9 (5)
Calf	3 $\pm$ 1 (5)
Horse	24 $\pm$ 3 (3)
Rabbit	79 $\pm$ 17 (3)
Hen	13 $\pm$ 3 (3)

activity in calf haemolysates (Table 1) could not be explained by TPP II activity, since Ser(P)-Val-Ala was not detectably formed (results not shown).

On incubation of hen haemolysates with the [³²P]phosphohexapeptide used in the standard assay, several ³²P-labelled cleavage products were detected when the incubation mixture was analysed by high-voltage electrophoresis (results not shown). This was opposite to the pattern obtained with haemolysates from most other species, where the main cleavage product is Ser([³²P]P)-Val-Ala, and indicates the existence of a population of aminopeptidases and/or endopeptidases with specificities partially different from those found in mammalian blood cells.

TPP II of liver homogenates of various species

The data of Table 2 shows that tripeptidyl peptidase II activities in the liver homogenates of all mammalian species analysed were of the same magnitude as the TPP II activity previously reported for rat liver, which was about 160 units/mg of wet tissue (Bålów *et al.*, 1983). In contrast with the absence of TPP II activity in calf erythrocytes, the liver of the same species seems to be a rich source of this peptidase.

High-voltage electrophoretic analysis of the cleavage products from the incubation with hen liver homogenates showed a pattern similar to that found with liver samples of animals.

Table 2. TPP activity in liver homogenates from different species

Homogenates were prepared as described in the Experimental section. Peptidase activity was measured by the standard assay and activity values refer to 1 g of wet tissue.

Species	Enzyme activity (units) [mean $\pm$ S.D. (n)]
Human	190 (1)
Hog	135 $\pm$ 39 (3)
Calf	219 $\pm$ 41 (4)
Rabbit	147 $\pm$ 41 (3)
Hen	97 $\pm$ 5 (3)

Affinity purification of rabbit antibodies against native TPP II and the peptidase 135000- M_r subunit from human erythrocytes

In a previous study evidence was provided that the catalytic site of TPP II, purified from human erythrocytes, resides in a subunit of M_r 135000 appearing as the main protein band on SDS/polyacrylamide-gel electrophoresis under reducing conditions (Bålów *et al.*, 1986). The IgG fraction from rabbits immunized with purified native enzyme was isolated on protein A-Sepharose and further affinity-purified against immobilized subunit and native peptidase. The specific antibodies recovered represented 2–6% of total IgG in both cases. As can be seen from Fig. 1 (lanes A and B), both antibody preparations recognized the 135000- M_r subunit of human TPP II transferred on to nitrocellulose after electrophoresis in SDS/polyacrylamide.

Cross-reactivity with TPP II from rat liver

When purified peptidase from rat liver was subjected to immunoblot analysis, anti-135 kDa cross-reacted with the 135000- M_r subunit of the liver enzyme (Fig. 1, lanes C and E). This antibody preparation was therefore chosen for an immunochemical screening of TPP II in different species. It is also apparent from Fig. 1 that the native

Fig. 1. Immunoblot analysis of purified TPP II from human erythrocytes and rat liver

Purified TPP II from both sources was electrophoresed on an 8%-(w/v)-polyacrylamide slab gel in the presence of 0.1% SDS, followed by electrophoretic transfer to nitrocellulose sheets and analysed for immune-reactive materials as described in the Experimental section. Lane A, human TPP II (1.1 μ g) incubated with anti-(TPP II) (0.8 μ g/ml); B, human TPP II (1.1 μ g) incubated with anti-135 kDa (1.1 μ g/ml); C, rat liver TPP II (1.1 μ g) incubated with same antibody preparation and dilution as in B; D and E, Coomassie Brilliant Blue-stained samples as in B and C respectively; F, reference proteins: myosin (M_r 200000), β -galactosidase (M_r 116250), phosphorylase b (M_r 92500), bovine serum albumin (M_r 66200) and ovalbumin (M_r 45000).

peptidase purified from both sources contains a minor portion of immunoreactive polypeptides of equal molecular size.

Immunoblot analysis of TPP I

Purified lysosomal TPP I from hog ovaries (M_r 57000 and acidic pH optimum; McDonald *et al.*, 1985) was not recognized by anti-135 kDa, as shown in Fig. 2, lane A. It is therefore assumed that TPP I would not interfere in the further immunoblot analysis.

Immunoblot analysis of haemolysates of various species

When calf haemolysates were analysed with immunoblot techniques, no immunoreactive material corresponding to the 135000- M_r polypeptide was detected. However, calf haemolysates contained an immunoreactive polypeptide of M_r approx. 84000 (Fig. 3, lane C). Polypeptides of this M_r were also seen on immunoblots of horse and rabbit haemolysates, in addition to a protein of M_r 105000 (Fig. 3, lanes D and E). Material of the latter size could also be detected in purified human and rat enzyme as well as in haemolysates of human erythrocytes (result not shown).

The diffuse spot with M_r 50000–55000 seen with the rabbit sample is attributed to H(heavy)-chains of rabbit IgG, detected by the goat anti-rabbit IgG-peroxidase conjugate used in the immunoblot analysis (Ahmed-Zadeh *et al.*, 1971). This interpretation was confirmed by incubations where the primary antibody was omitted (result not shown).

All immunoblot experiments described in the present work were also performed with rabbit pre-immune serum, whereby no detectable staining was seen, except from the H-chains in samples of rabbit erythrocytes and liver.

Fig. 2. Immunoblot analysis of purified TPP I and II

Purified TPP I of pregnant hog ovaries and purified TPP II of human erythrocytes were subjected to polyacrylamide electrophoresis and immunoblot analysis as described in the Experimental section. The antibodies used were anti-135 kDa at 1.1 μ g/ml. Lane A, TPP I (8 μ g); B, TPP II (0.7 μ g).

Fig. 3. Immunoblot analysis of TPP II in haemolysates from different species

Polyacrylamide-gel electrophoresis and immunoblot analysis were performed as described in the Experimental section. The antibodies used were anti-135 kDa at 1.1 $\mu\text{g}/\text{ml}$. The lanes contained haemolysates from: A, monkey (2.6 mg of protein); B, hog (3.7 mg of protein); C, calf (2.6 mg of protein); D, horse (2.9 mg of protein); E, rabbit (2.6 mg of protein); F, hen (2.2 mg of protein); and G, purified human TPP II (0.7 μg).

Fig. 4. Immunoblot analysis of TPP II in liver homogenates from different species

Polyacrylamide-gel electrophoresis and immunoblot analysis were performed as described in the Experimental section. The antibodies used were anti-135 kDa at 1.1 $\mu\text{g}/\text{ml}$. Lanes contained liver homogenates from: A, human (270 μg of protein); B, hog (120 μg of protein); C, calf (135 μg of protein); D, rabbit (250 μg of protein); E, hen (120 μg of protein); and F, purified human TPP II (0.7 μg).

Immunoblot analysis of liver homogenates of various species

Immunoblot analysis of human liver gave rise to two bands of M_r 135000 and 40000 respectively (Fig. 4, lane A). The polypeptide with the lower M_r may correspond to a fragment of similar size seen on immunoblots of purified human peptidase. The 135000- M_r polypeptide was detected also in calf liver homogenates (Fig. 4, lane C), as well as in samples from rabbit, hog and hen (Fig. 4, lanes B, D, and E), a finding that is compatible with the presence of TPP II activity in the livers of these species.

TPP II activity of rat reticulocytes and *E. coli* extracts

The TPP II activities in reticulocytes and *E. coli* were of special interest, since numerous studies of the ATP-dependent protein degradation (Etlinger & Goldberg, 1977; Daniels & Hipkiss, 1978; Botbol & Scornik, 1979; Murakami *et al.*, 1979; Voellmy & Goldberg, 1981) and the ATP-ubiquitin-mediated proteolytic pathway (Ciechanover *et al.*, 1978; Hough & Rechsteiner, 1984) have been performed with these cells in several laboratories. Since the content of reticulocytes in blood from newborn rats ranges between 80 and 95% (Creskoff *et al.*, 1949), this source was chosen for the preparation of reticulocytes as described in the Experimental section. The TPP II activity in rat reticulocytes was comparable with the levels in blood from adult rats, and immunoblot analysis revealed the presence of immunoreactive polypeptides of M_r 135000 and 84000 (Fig. 5, lane A).

Fig. 5. Immunoblot analysis of TPP II in rat reticulocyte lysate and *E. coli* extract

Polyacrylamide-gel electrophoresis and immunoblot analysis were performed as described in the Experimental section. The antibodies used were anti-135 kDa at 1.1 $\mu\text{g}/\text{ml}$. Lane A, rat reticulocyte lysate (1.6 mg of protein); lane B, *E. coli* extract (0.2 mg of protein); and lane C, purified human TPP II (0.7 μg).

When extracts of *E. coli* were analysed for peptidase activity under the conditions of the standard assay of TPP II, no enzyme activity could be detected. Interestingly, however, as can be seen in Fig. 5, lane B, immunoblot analysis showed immunoreactive polypeptides with M_r values of about 135000 and 40000. Despite the quite weak reaction seen with the bacterial extracts, the reaction was considered to be specific, since no immunoreactive material was detected either upon incubation with pre-immune serum or on omission of the primary antibodies (results not shown).

DISCUSSION

In the immunoblot analyses of the present work, the rabbit antibodies used were affinity-purified against the denatured subunit of M_r 135000 of TPP II of human erythrocytes. Therefore any polypeptide of M_r lower than 135000 detected by these antibodies is considered to be attributable to epitopes common to those of the 135000- M_r subunit.

On immunoblot analysis of the purified peptidase of human erythrocytes, several trace immunoreactive polypeptides with M_r values of less than 135000 were detected. These polypeptides may be due to a proteolytic digestion of the enzyme, either in the intact erythrocytes or during the purification of the enzyme. Alternatively, the native enzyme preparation may contain minor subunits or impurities with epitopes common to those in the dominating 135000- M_r subunit. The former explanation is partially supported by the facts that some trace polypeptides of the purified erythrocyte enzyme incorporated ^3H -labelled di-isopropyl fluorophosphate, and that this incorporation was inhibited by *p*-chloromercuribenzoate, which inhibits the peptidase activity (Bålów *et al.*, 1986). An evaluation of the latter possibility will probably require the development of methods that will permit the reversible dissociation of the native enzyme.

The present results show that cell extracts which manifest TPP II activity also contain an immunoreactive polypeptide of M_r 135000. The only mammalian cell type so far investigated where TPP II activity is absent is bovine erythrocytes. These haemolysates also lacked the immunoreactive 135000- M_r polypeptide. Haemolysates of human, monkey, horse and rabbit contained an additional immunoreactive polypeptide of M_r ~105000. A polypeptide of this size could also be seen on immunoblots of purified enzyme from both human erythrocytes and rat liver, which suggests that this polypeptide is a fragment of the 135000- M_r subunit. A similar interpretation must obviously also be considered for the immunoreactive polypeptide of M_r ~84000 in haemolysates of calf and horse. The derivation of the 84000- M_r polypeptide in calf haemolysates from TPP II requires that the enzyme was present in a precursor of the mature erythrocyte, which seems possible, since the peptidase is present in the liver of the same species.

A general conclusion of the present work is that the

occurrence of TPP II is widespread, although not ubiquitous, and that epitopes found in the human enzyme have been highly conserved during evolutionary development. Nevertheless, no cross-reaction between human TPP II and hog TPP I could be detected by immunoblot analysis. A detailed explanation of the existence of several immunoreactive bands of M_r < 135000 in the purified peptidase, as well as in cell extracts from several species, apparently has to await a comparison of the structure of the 135000- M_r subunit with those of the immunoreactive polypeptides.

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