Supramolecular structure of tripeptidyl peptidase II from human erythrocytes as studied by electron microscopy, and its correlation to enzyme activity

Elizabeth MACPHERSON,* Birgitta TOMKINSON,†‡ Ros-Mari BÅLÖW,† Stefan HÖGLUND and Örjan ZETTERQVIST†

*Department of Biochemistry and †Department of Medical and Physiological Chemistry, University of Uppsala, Biomedical Center, S-751 23 Uppsala, Sweden

Tripeptidyl peptidase II is an extralysosomal serine peptidase of an unusually large size, i.e. M_r greater than 10^6 for the native enzyme and M_r 135 000 for the subunit. The enzyme from human erythrocytes was studied by electron microscopy on samples negatively stained by ammonium molybdate. Two different structural representations of the purified enzyme were obtained, both with a length of about 50 nm, and consisting of repetitive substructures. Upon dialysis of the enzyme against a Tris/HCl buffer, the activity was gradually decreased. This decrease was shown to parallel the dissociation of the large enzyme structures into smaller ones, the smallest measuring 3 nm by 10 nm and apparently corresponding to the repetitive substructures. The results indicate that a large polymeric form of the enzyme is a prerequisite for full activity.

INTRODUCTION

Tripeptidyl peptidase II (TPP II), an extralysosomal exopeptidase originally detected in rat liver [1], is present in other tissues in the rat [2] as well as in liver and erythrocytes of several other species [3]. The enzyme, which has been purified essentially to homogeneity from both rat liver and human erythrocytes [2], has a large native M_r (greater than 10^6) as judged by gel filtration [1], and a subunit M_r of 135 000 [2] in polyacrylamide-gel electrophoresis in the presence of SDS under reducing conditions. The enzyme is inhibited by di-isopropyl phosphorofluoridate, whereby the subunit binds approx. 1 mol of di-isopropyl phosphate/mol of subunits [2], which indicates that the subunits are of only one type.

In order to correlate the enzyme activity and macromolecular structure of this unique peptidase, various pilot experiments were made to dissociate the enzyme and study its behaviour in gel filtration. However, losses of enzyme activity invariably ensued, and it was not possible to determine whether these were due to dissociation, aggregation or irreversible adsorption on the gel. Therefore, the finding of conditions for obtaining clear and reproducible images of the enzyme by electron microscopy on negatively stained enzyme samples, reported in the present paper, offered a new opportunity for the study of the correlation between activity and structure.

Some of these data have been presented in a preliminary form [4,5].

EXPERIMENTAL

Materials

Colloidin solution (4 %, w/v) was obtained from Ladd Research Industries (Burlington, VT, U.S.A.). Grids and ammonium molybdate came from Agar Aids (Stansted,

Essex, U.K.). Ala-Ala-Phe 4-methyl-7-coumarylamide was purchased from Bachem Feinkemikalien AG (Bubendorf, Switzerland). Calibration proteins for gel electrophoresis were bought from Bio-Rad Laboratories (Richmond, CA, U.S.A.) (high- M_r protein standards) and crystallized bovine serum albumin from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glycerol was obtained from Dow Chemicals (Detroit, MI, U.S.A.) and met U.S.P. requirements. All other reagents were of analytical grade. TPP II was prepared essentially as previously described [2], from outdated human erythrocyte concentrates, which were obtained from the blood bank at the University Hospital of Uppsala.

Assay of TPP II activity

Activity measurements were performed at 30 °C in a mixture of 2.5 ml of 100 mM-potassium phosphate buffer, pH 7.5, containing 2 mM-dithiothreitol and 0.1 mM-Ala-Ala-Phe 4-methyl-7-coumarylamide as substrate. The reaction was started by the addition of enzyme and monitored by measuring the change in absorbance at 365 nm. The rate was calculated by use of a molar absorption coefficient of $16200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [6].

Dialysis of TPP II

TPP II [30 µg in 50 µl of 50 mM-potassium phosphate buffer, pH 7.5, containing 30 % (w/v) glycerol and 1 mM-dithiothreitol] was dialysed at 4 °C against 100 ml of 2 mM-Tris/HCl buffer, pH 8.0, containing 3 % (w/v) glycerol and 0.5 mM-2-mercaptoethanol (Tris dissociation buffer), in an open plastic tube (diameter 11 mm) with one end covered with a dialysis membrane. Before dialysis and after various intervals, as indicated in Table 1 and Figs. 2 and 3, 5 µl samples were withdrawn, diluted with the Tris dissociation buffer to a final protein concentration of 0.08 mg/ml and applied on electron-

Abbreviation used: TPP II, tripeptidyl peptidase II.

‡ To whom correspondence should be addressed.

Fig. 1. Electron micrograph of TPP II

Peptidase was diluted in 20 mM-sodium phosphate buffer, pH 7.4, containing 1 M-NaCl to a final protein concentration of 0.02 mg/ml, and electron-microscopy grids were prepared as described in the Experimental section. (a) Overall view of purified human erythrocyte TPP II. (b) Inset showing enlargement from (a) with 1, double-bow structures of two different kinds, and 2, an enzyme structure with a single-bow-like appearance. Bars indicate 100 nm.

Fig. 2. Electron micrographs of dialysed TPP II

TPP II was dialysed against the Tris dissociation buffer as described in the Experimental section. The structures were divided into four different size classes: 1, structures containing 14–18 'limiting units'; 2, structures containing seven to nine units; 3, structures containing four to six units; 4, structures containing one to three units. (a) Before dialysis; (b) after dialysis for 1 h; (c) after dialysis for 2 h; (d) after dialysis for 4 h. The numbered arrowheads indicate examples of the different size classes. Bars indicate 100 nm.

Table 1. Distribution of TPP II between different size classes as a function of dialysis time

TPP II was dialysed against the Tris dissociation buffer and after the times indicated samples were taken for electron microscopy and activity measurements as described in the Experimental section. With the use of $3\times$ enlargements of the micrographs, a $4\text{ cm} \times 5\text{ cm}$ area was placed over 12 non-overlapping positions for each dialysis time, and the number of copies of each of four different size classes, defined in the legend to Fig. 2, were counted. In order to obtain an estimate of the total number of 'limiting units' in each class, the number of structures in each class was multiplied by the average number of limiting units in each structure. The fractional distribution of limiting units present in each size class was calculated.

Dialysis time (h)	Size class ...	Number (N) of structures and fractional distribution (%) of limiting units in each size class							
		1		2		3		4	
		N	%	N	%	N	%	N	%
0		114	24	329	34	414	27	570	15
1		74	20	48	7	176	15	1663	58
2		43	13	27	4	164	15	1817	68
4		6	2	10	2	71	8	2019	88

microscopy grids, prepared as described below. At the same time $2\text{ }\mu\text{l}$ samples were taken for activity measurements in the assay described above. As a result of osmotic effects, the sample became diluted 2-fold during the initial few minutes of dialysis. This dilution was taken into account in the calculation of the enzyme activity.

Electron microscopy

To prepare the grids for electron microscopy, a drop of 4% (w/v) colloidin solution from a Pasteur pipette was applied on to a 10 cm-diameter glass dish filled with distilled water. The colloidin film was allowed to dry for 3 min. Copper grids (3 mm diameter and 400 mesh) were placed on the film (grey appearance by interference pattern) and picked up with the help of Parafilm. Only freshly prepared grids (3–48 h) were used. A 5–10 μl sample was applied for 2 min on to the grid and then excess material was blotted out with filter paper. The grids were negatively stained with 2% (w/v) ammonium molybdate in 0.05 M-ammonium acetate buffer, pH 7.5. After 45 s, excess stain was blotted out and the sample was allowed to dry. Grids were examined in a Philips 300 electron microscope at 60 kV and micrographs were taken at a magnification of $25000\times$.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed in the discontinuous SDS system of Laemmli [7] as described by O'Farrell [8]. The acrylamide concentration of the running gel was 8% (w/v). Before application, the samples were made 2.3% (w/v) in SDS, 5% (v/v) in 2-mercaptoethanol and 10% (w/v) in glycerol, then heated in a boiling-water bath for 3 min. After electrophoresis, the gel was treated for 1 h with 10% (w/v) trichloroacetic acid and stained and destained as described in ref. [9].

RESULTS AND DISCUSSION

Electron-microscopic images of TPP II were obtained by using ammonium molybdate as a suitable contrast agent [10]. Fig. 1 shows two different structural representations, both with a length of about 50 nm. One type of structural image is a single bow-like structure about

Fig. 3. Correlation between activity and structure of TPP II

The enzyme was dialysed against the Tris dissociation buffer and after the indicated times samples were taken for activity measurements (○) and electron microscopy (●) as described in the Experimental section. The values of relative activity represent means $\pm$ s.d. for three different experiments. The mean of activity before start of dialysis was $15.6 \pm 1.1\text{ }\mu\text{mol/min per mg}$. The fraction of TPP II subunits present in size classes 1 + 2, defined in the legend to Fig. 2 and calculated as described in the legend to Table 1, is normalized to the value (58%) at zero time.

10 nm wide. The other type of image is interpreted as consisting of two of the single bows, which intersect, or resemble an ellipse. The maximum width is about 20 nm and 24 nm respectively. All of the structural images appear to consist of a number of smaller units, labelled 'limiting units', each of which is about 3 nm by 10 nm. There seem to be seven to nine limiting units in the single bow-like structure and 14–18 in the double-bow structures. Whether these limiting units really correspond to the 135000- M_r subunit of the enzyme, or a multiple

Fig. 4. Polyacrylamide-gel electrophoresis of TPP II before and after dialysis

Samples of undialysed TPP II and TPP II dialysed for 4 h against the Tris dissociation buffer were prepared and loaded on to an 8% polyacrylamide slab gel, all as described in the Experimental section. Lane 1, undialysed TPP II (1.0 μ g); lane 2, TPP II dialysed for 4 h (1.0 μ g); lane 3, reference proteins: myosin (M_r 200 000), β -galactosidase (M_r 116 250), phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 200) and ovalbumin (M_r 45 000).

thereof, is not known at the present. Still, it seems reasonable to conclude that the previous estimate [1] of M_r greater than 10^6 for the active enzyme is compatible with the present data.

During attempts to find conditions to modulate the activity of the enzyme, it was found that dialysis against Tris dissociation buffer caused a gradual decrease in activity. In order to correlate this change in activity with possible changes of the macromolecular structure of the

enzyme, electron-microscopic and enzyme activity analyses were performed on samples withdrawn after different periods of dialysis. It is evident from Fig. 2 and Table 1 that the dialysis causes a dissociation of the enzyme structure. The dissociation is correlated with the decrease in enzymic activity (Fig. 3). This decrease is not due to a major proteolytic cleavage during dialysis, as no difference in subunit size could be seen upon polyacrylamide-gel electrophoresis in the presence of SDS between undialysed and dialysed TPP II (Fig. 4). The decrease in enzymic activity is considered to be due mainly to the decrease in glycerol concentration during the dialysis. If the enzyme is dialysed for 4 h at 4 °C against the Tris dissociation buffer containing 30% (w/v) glycerol, the enzyme retains 74% of its activity (results not shown) compared with 15% if the buffer contained 3% glycerol (Fig. 3).

Two conclusions emerge from this study. First, the enzyme seems to be represented by a few ordered polymeric forms. Secondly, a polymeric form is a prerequisite for a fully active enzyme. This implies that the enzyme may be regulated by control of the polymerization.

We thank Åsa Flygh and Titti Rubinstein for skilful technical assistance in preparing TPP II. This work was supported by the Swedish Research Council (Project 13X-04485) and the Medical Faculty of Uppsala.

REFERENCES

1. Bålöw, R.-M., Ragnarsson, U. & Zetterqvist, Ö. (1983) *J. Biol. Chem.* **258**, 11622–11628
2. Bålöw, R.-M., Tomkinson, B., Ragnarsson, U. & Zetterqvist, Ö. (1986) *J. Biol. Chem.* **261**, 2409–2417
3. Bålöw, R.-M. & Eriksson, I. (1987) *Biochem. J.* **241**, 75–80
4. Macpherson, E., Bålöw, R.-M., Höglund, S., Tomkinson, B. & Zetterqvist, Ö. (1986) *J. Ultrastruct. Res.* **94**, 268
5. Bålöw, R.-M. (1986) *Acta Univ. Ups. Abstr. Uppsala Diss. Fac. Med.* **56**, 26–29
6. Saifuku, K., Sekine, T., Namihisa, T., Takahasi, T. & Kanaoka, Y. (1978) *Clin. Chim. Acta* **84**, 85–91
7. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
8. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
9. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851
10. Munn, E. A. (1974) *Methods Enzymol.* **32**, 20–35

Received 17 March 1987/8 June 1987; accepted 10 August 1987