A distinct thimet peptidase from rat liver mitochondria

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Thimet peptidase has been purified from rat liver mitochondria and found to share the characteristics of a thiol-dependent metallo-endopeptidase previously described for an enzyme in the cytosolic fraction from rat testis: inhibition by EDTA, reactivation by Zn^{2+}, requirement of dithiothreitol for maximal and stable activity, and inhibition by *N*-ethylmaleimide. The K_i for inhibition by *N*-[1-(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoic acid is 2.6 μM, 100-fold higher than the value for the cytosolic form. The mitochondrial form is not inhibited by antisera against the cytosolic form, and the two forms of the enzyme show quantitative differences in substrate specificity. The name thimet peptidase II is suggested for the enzyme from rat mitochondria.

Thimet peptidase; Pz-peptidase; Endo-oligopeptidase A; Soluble metallo-endopeptidase; Mitochondrial enzyme

1. INTRODUCTION

Thimet peptidase (EC 3.4.24.15), previously known as Pz-peptidase, endo-oligopeptidase A [1] and soluble metallo-endopeptidase [2] is a thiol-dependent metallo-endopeptidase [3] that acts on oligopeptides [4,5]. Most published reports have concerned the cytosolic form of the enzyme, but Heidrich et al. [6] discovered a 'Pz-peptidase' in highly purified mitochondria from rat liver.

It has previously been shown that the species variants of thimet peptidase from rat and rabbit differ in properties such as affinity for the inhibitor Cpp-Ala-Ala-Phe-pAB [3], but we now show that different forms also occur within a single species. In this report we compare the thimet peptidase from rat liver mitochondria with the better-known form of the enzyme from rat testis cytosol.

2. EXPERIMENTAL

2.1. *Enzyme assays*

Activity on all substrates was assayed in 50 mM Tris/HCl, 0.1 mM dithiothreitol, 0.05% Brij 35, pH 7.8, at 30°C. Normally the quenched fluorescence substrate Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) was used as substrate at a 10 μM concentration [7]. Where indicated, an alternative quenched fluorescence substrate, Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys [8] was used under the same assay conditions. The fluorimeter was controlled, and data collected, by an IBM-compatible computer using FLUSYS software [9].

For the hydrolysis of Bz-Gly-Ala-Ala-Phe-pAB (1 mM), phosphoramidon and microsomal alanyl aminopeptidase (EC 3.4.11.2) (Sigma No L-5006) were included in the assay in final concentrations of 25 μM and 0.1 mg/ml, respectively. After a 30 min incubation, the reaction was stopped with trichloroacetic acid and the assay was completed as described [10].

Assays involving Pz-Pro-Leu-Gly-Pro-D-Arg were done as previously described [11] with a substrate concentration of 320 μM.

For the hydrolysis of bradykinin, the substrate (70 μM) was incubated with enzyme for 10 min. The degradation products were separated from the unhydrolysed bradykinin by high pressure liquid chromatography (HPLC) on a Technopak 10 C18 column with a gradient (5–50%) of (i) 0.1% phosphoric acid and (ii) acetonitrile, with a flow rate of 1.5 ml/min and detection at 220 nm. Data were processed with the Varian Vista 402 system. The same HPLC system was used to analyse the products of hydrolysis of Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp), with amino acid analysis as described [7].

For each assay, one unit of activity was defined as that hydrolysing 1 μmol of substrate per min at 30°C.

2.2. *Protein assay*

The Bio-Rad assay was used with bovine serum albumin as standard [12].

2.3. *Enzyme purification*

For the purification of mitochondrial thimet peptidase, rat liver was homogenized in 3 vol of 0.24 M sucrose, 50 mM Tris/HCl, pH 8.0. After a debris spin of 5 min at 1000 × *g*, the supernatant was centrifuged for 20 min at 10 000 × *g*. The resulting mitochondrial pellet was washed in the sucrose/Tris buffer and resuspended in 50 mM Tris/HCl, pH 8.0, 2% (w/v) Triton X-100. After 30 min at 4°C, the preparation was centrifuged for 20 min at 10 000 × *g* and the supernatant was run on columns of DEAE-cellulose, Sephacryl S-200 HR, Mono Q and Mono P as previously described for the rat testis enzyme [3], except that 0.2% Brij 35 was included in all buffers after the DEAE-cellulose step.

The cytosolic form of thimet peptidase was purified from rat testis as previously described [3].

In some experiments, the 10 000 × *g* supernatant of the liver preparation was used and is referred to as liver cytosol.

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Abbreviations: Bz, α-*N*-benzol; Cpp, *N*-[1(*RS*)-carboxy-3-phenylpropyl]; Dnp, 2,4-dinitrophenyl; Mcc, 7-methoxycoumarin-3-carboxylyl; pAB, *p*-aminobenzoic acid; Pz, *N*,α(4-phenylazo)benzyloxycarbonyl

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2.4. *SDS-polyacrylamide gel electrophoresis*

Purified rat liver mitochondrial thimet peptidase was run reduced in an SDS/polyacrylamide gel (total acrylamide concentration 5% (w/v) as described by Bury [13].

2.5. *Analytical gel chromatography*

Purified mitochondrial thimet peptidase was run on an LKB Ultropac TSK-G 3000 SW column in 50 mM Tris/HCl, 200 mM NaCl, 0.05% Brij 35, 0.1 mM dithiothreitol, pH 7.2, at 0.25 ml/min. The fractions were assayed for activity. As M_r standard, transferrin was run under the same conditions.

2.6. *Metal- and thiol-dependence*

Inhibition by EDTA, reactivation by $ZnCl_2$ and inhibition by *N*-ethylmaleimide were assayed as described [3].

2.7. *Kinetic data*

Values for K_m and K_i (corrected for competition by substrate) were obtained as previously described [3,7].

2.8. *Raising of antisera*

Thimet peptidase purified from rat testis (100 μg) in complete Freund's adjuvant was injected intramuscularly in two rabbits. Further injections of 50 μg of antigen in incomplete Freund's adjuvant were made subcutaneously 3 and 6 weeks later. Antisera were collected one week after the last injection.

3. RESULTS AND DISCUSSION

3.1. *Purification of the mitochondrial enzyme*

The rusults of the purification procedure for the mitochondrial thimet peptidase are summarized in Table I. Two peaks of activity were eluted from the Mono Q column, at 140 mM and 165 mM NaCl, respectively. On the Mono P chromatofocusing column, activity from the 140 mM NaCl peak was eluted at pH 5.2 and that from the 165 mM peak at pH 4.9.

Table I

Purification of thimet peptidase from rat liver mitochondria

Purification step	Protein (mg)	Activity (mU)	Purification factor	Yield (%)
Solubilised mitochondria	5200	7215	(1)	(100)
DEAE-cellulose chromatography	176	4905	20	68
Sephacryl S-200 HR chromatography	23.5	1314	40	18
Mono Q HPLC peak 1	1.3	216	120	3.0
Mono Q HPLC peak 2	1.9	241	92	3.3
Mono P HPLC peak 1	0.15	79	382	1.1
Mono P HPLC peak 2	0.14	59	305	0.8

115 g of rat liver was used as described in the text.

We assume that these are approximately the isoelectric points of the two isoenzymes. Heidrich et al. [6] reported an isoelectric point of 5.1 and failed to detect multiple isoenzymes. The value of p*I* for the rabbit muscle cytosolic form was 4.8 [11].

The pH 5.2 isoform of the rat liver mitochondrial enzyme was used for further characterisation. In SDS/polyacrylamide gel electrophoresis the final product ran as two bands with apparent M_r values of 90 000 and 74 000. In analytical gel chromatography, enzymic activity was only detectable in the fractions eluting later than transferrin (M_r 78 000), corresponding to the lower M_r band on the SDS/polyacrylamide gel. We conclude that the 74 000 band represents the active enzyme and the 90 000 band is a contaminant.

In contrast, Heidrich et al. [6] reported the M_r of the mitochondrial enzyme to be between 93 000 and 98 000 on the basis of SDS electrophoresis, analytical ultracentrifugation and gel chromatography. The difference may be due to the different methods used. Our value of 74 000 for the mitochondrial enzyme is identical with that of the cytosolic form rat testis [3] and rabbit muscle [11].

3.2. *Thiol-dependence*

Maximal, stable activity on Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) was observed in the presence of 0.1 mM dithiothreitol. In a continuous rate assay started without dithiothreitol, 89% of the activity was restored by the addition of 0.1 mM dithiothreitol after a 5 min incubation at 30°C, but only 67% of the activity was restored when dithiothreitol was added after 20 min. Prolonged incubation in the absence of dithiothreitol inactivated the enzyme irreversibly. The enzyme was also completely inactivated by exposure to 0.5 mM *N*-ethylmaleimide for 1 min.

3.3. *Metal-dependence*

Activity of the mitochondrial thimet peptidase was completely abolished by incubation in 10 mM EDTA at 4°C for 24 h. After dilution of the EDTA, activity was restored (93%) by 100 μM $ZnCl_2$.

Table II

Relative rates of hydrolysis of substrates by the mitochondrial and cytosolic forms of thimet peptidase

Substrate	Mitochondrial form	Cytosolic form
Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp)	(1)	(1)
Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys	0.06	1.0
Bz-Gly-Ala-Ala-Phe-pAB	0.10	0.56
Pz-Pro-Leu-Gly-Pro-D-Arg	0.68	0.31
Bradykinin	1.6	1.5

Assay conditions were as described in section 2. The results have been normalised with reference to the activity of Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp).

Table III

Michaelis constants for the hydrolysis of quenched fluorescence substrates by the mitochondrial and cytosolic forms of thimet peptidase

Substrate	Mitochondrial form K_m (μM)	Cytosolic form K_m (μM)
Mcc-Pro-Leu-Gly-Pro-D-Lys-(Dnp)	23.8	6.4
Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys	15.4	11.3

Data were obtained in continuous rate assays as described in section 2 and were corrected for the self-absorption of Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp).

These data are in good agreement with the report of Heidrich et al. [6] on the influence of EDTA and *N*-ethylmaleimide on the mitochondrial enzyme, and indicate that it is metal- and thiol-dependent, like the cytosolic forms from rabbit muscle and rat testis [3].

3.4. *Substrate specificity*

The purified mitochondrial enzyme hydrolysed the substrates shown in Table II, all of which are also hydrolysed by the cytosolic form. However, the relative activities of the two forms of enzyme are different, as are the Michaelis constants (Table III). Analysis of the products of cleavage of Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) by the mitochondrial enzyme by HPLC showed products identical to those produced by the cytosolic form, which cleaves the Leu-Gly bond [7].

3.5. *Inhibition characteristics*

Corrected values of K_i for the inhibitor Cpp-Ala-Ala-Phe-pAB [14] were found to be 2.6 μM and 0.035 μM, respectively, for the purified mitochondrial form and crude cytosolic form of the enzyme from rat liver. These values may be compared to that of 0.017 μM for the purified cytosolic enzyme from rat testis [2], and we consider that the values for the two cytosolic forms are identical within the limits of experimental error.

3.6. *Immuno-inhibition*

Antisera raised against rat testis thimet peptidase in two rabbits did not affect the rate of hydrolysis of Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) by the mitochondrial form of the enzyme, but both inhibited the cytosolic form purified from rat testis by 90%, when used at 50 μl/2.5 ml incubation volume. Activity present in the liver cytosol was also inhibited by the antisera.

4. CONCLUSIONS

Two forms of thimet peptidase are present in rat tissues. The properties of these have been compared primarily by use of the purified forms from liver mitochondria and testis cytosol, but the liver cytosol form is similar to that of testis, suggesting that the distinct mitochhondrial and cytosolic forms are not tissue variants, but may be present in rat tissues generally. The two forms differ in substrate specificity, inhibitor sensitivity and inhibition by antisera, and are therefore almost certainly products of distinct genes. However, the two forms are closely similar in physicochemical properties, and share the unusual characteristic of being thiol-dependent metalloendopeptidases. It is therefore quite probable that the genes are homologous. To distinguish the mitochondrial from the cytosolic form, the name thimet peptidase II is suggested for the enzyme from rat mitochondria.

In view of the existence of at least two distinct forms of thimet peptidase in the rat, it will be necessary to characterize the enzyme from different locations, including brain synaptosomal membranes [15], with care.

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