Identification, purification and partial characterization of a carboxypeptidase from the matrix of rat liver mitochondria: a novel metalloenzyme

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A novel carboxypeptidase has been purified to apparent homogeneity from the matrix fraction of rat liver mitochondria by using a procedure mainly based on immobilized-metal-affinity chromatography (IMAC). This carboxypeptidase has been named mCP-III, since it represents the third major peak of carboxypeptidase activity after the IMAC step of purification. mCP-III hydrolyses a number of N-blocked dipeptides, with preference for Cbz-Phe-Ala, and shows no degrading activity towards ^{125}I -casein. The optimal pH of its activity is 7.6, the

apparent K_m for Cbz-Phe-Ala is 0.12 mM and the specific activity is 145.5 $\mu\text{mol}/\text{min}$ per mg of protein. The enzyme is a typical metalloproteinase, is inhibited by 1,10-phenanthroline and carboxypeptidase inhibitor and re-activated by added Zn^{2+} and Co^{2+} . The molecular mass estimated by molecular-sieve h.p.l.c. was approx. 115 kDa with two protein bands of 61 and 50 kDa shown by SDS/PAGE analysis, indicating that the enzyme is active as a dimer. This is the first clearly identified carboxypeptidase within mitochondria.

INTRODUCTION

Despite the current interest in intracellular proteinases, the proteolytic systems of mitochondria remain poorly defined. In recent years some proteinases having particular characteristics have been localized in this organelle: an ATP-dependent endopeptidase [1], Ca^{2+} -activated endopeptidases [2], and chelator-sensitive aminopeptidases and endopeptidases [3–7].

As previously demonstrated in our laboratory, the exopeptidase activity of rat liver mitochondria is mainly located in the matrix fraction [8]. Another interesting observation concerns the fact that most of the proteolytic activity in the mitochondrial matrix is abolished by chelating agents [9]. Considering that many exopeptidases, namely the animal carboxypeptidases, are metalloenzymes [10], it can be assumed that the mitochondrial matrix of rat liver possess a number of carboxypeptidases. Therefore we have focused our attention on this latter type of enzyme. The purpose of the present investigation was to identify the carboxypeptidases in the mitochondrial matrix of the rat liver cell. Here we report the purification and partial characterization of mCP-III, the most significant of these mitochondrial carboxypeptidases. The molecular mass, substrate preference, pH-dependence and susceptibility to various metalloproteinase inhibitors have been examined.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade, and were purchased from Sigma (St. Louis, MO, U.S.A.) unless stated otherwise. DE-52 was from Whatman Biosystems Ltd., and the f.p.l.c. Chelating Superose HR 10/2 was from Pharmacia (Uppsala, Sweden). αS1 -casein was iodinated with ^{125}I from Amersham International, using the chloramine-T method [11].

Animals and organ perfusion

Male Wistar rats (10 weeks old) were starved overnight before

being killed by decapitation, and their livers were immediately perfused until free of blood with warm saline (0.9% NaCl) pumped at 15 ml/min through the vena porta.

Preparation of mitochondria and submitochondrial fractions

Mitochondria were isolated and purified by our modification of the classical methods [12], using a low concentration of digitonin (10 $\mu\text{g}/\text{mg}$ of mitochondrial protein) that was shown to be very effective in purifying the mitochondria from adsorbed lysosomes without impairing subsequent fractionation. From these purified mitochondria, mitoplasts were obtained by a conventional method [13] that uses 0.12 mg of digitonin/mg of mitochondrial protein to disrupt the outer membrane. The mitoplasts (matrix plus inner membrane) were then separated by centrifugation for 15 min at 12000 g . In order to detach the matrix from the inner membrane, the mitoplasts were sonicated and centrifuged for 60 min at 144000 g . This was done in the presence of a cocktail of proteinase inhibitors [1 mM 1,10-phenanthroline, 0.1 mM dichloroisocoumarin (DCI), 50 $\mu\text{g}/\text{ml}$ E-64, 50 $\mu\text{g}/\text{ml}$ pepstatin, 50 μM bestatin]. The 12000 g supernatants were further centrifuged for 60 min at 144000 g for separation of the outer membrane and intermembrane space.

The proteins of the mitochondrial matrix were fractionated by two steps of ammonium sulphate precipitation, 0–30% and 30–90%. The matrix proteins precipitating between 30 and 90% were used as the enzyme source, the pellets being stored at -20°C .

DEAE-cellulose chromatography

The 30–90% ammonium sulphate precipitate was resuspended, filtered on a PD-10 column and applied to a DE-52 column exchanger (60 mm $\times$ 18 mm) previously equilibrated with 50 mM Mops, pH 7.8. The column was washed with buffer until the A_{280} of the eluate fell below 0.05. The adsorbed proteins were then eluted with 100 ml of a NaCl linear gradient (0–350 mM). The flow rate was 40 ml/h and all operations were carried out at 4°C .

Abbreviations used: IMAC, immobilized-metal-affinity chromatography; Bz, benzoyl; Cbz, carboxybenzoyl; DCI, dichloroisocoumarin; E-64, *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane; CPI, carboxypeptidase inhibitor from potato; IDA, iminodiacetic acid; TED, tris(carboxymethyl)-ethylenediamine.

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Immobilized-metal-affinity chromatography (IMAC)

An iminodiacetic acid (IDA)–Superose f.p.l.c. column (Chelating Superose HR 10/2; Pharmacia) was washed with distilled water and loaded with the metal by applying 5 ml of a 20 mM solution of CuCl_2 . Since elution of protein from the column was performed using an imidazole gradient, a previous run with this same imidazole gradient was necessary to ensure that no leakage of metal occurred during the experimental run. Before applying the protein sample, the column was washed with equilibrating buffer containing 50 mM Mops, 500 mM NaCl and 1 mM imidazole, pH 7.8. Pooled eluates of carboxypeptidase activity from a DE-52 column were made 500 mM in NaCl and 1 mM in imidazole, and then applied to the Cu^{2+} –IDA column. The column was washed with 10 ml of 5 mM imidazole and then eluted with a 30 ml imidazole linear gradient (5–20 mM). The flow rate was 60 ml/h, fractions of 1 ml were collected and all operations were carried out at 4 °C. If the enzyme aliquot was required for metal titration, the pooled eluates corresponding to a peak of activity from this direct IMAC were further processed by reversed IMAC on a metal-free tris(carboxymethyl)ethylenediamine (TED)-column prepared according to the procedure described by Porath and Olin [14], equilibrated and eluted with buffer containing 50 mM Mops and 500 mM NaCl, pH 7.8.

Gel filtration

Enzyme samples from IMAC were concentrated to about 500 μl in a Centricon (Amicon Co.) and injected into a Superose 12 HR 10/30 column (Pharmacia) eluted with 50 mM Mops and 150 mM NaCl, pH 7.8, at a flow rate of 300 $\mu\text{l}/\text{min}$. Glycerol (20%) was immediately added to the eluted fractions.

Enzyme assays and determination of kinetic parameters

Lactate dehydrogenase activity was assayed by the method of Beisenherz et al. [15], cytochrome *c* reductase by the method of Williams and Hamin [16], and acid phosphatase by the method of Torriani [17].

Subfractionation of mitochondria was routinely confirmed by measuring the distribution of marker enzymes (malate dehydrogenase, succinate dehydrogenase, adenylate kinase and monoamine oxidase) at the four structural levels of the organelle, assayed as described in [9].

Carboxypeptidase activities were tested towards a number of N-blocked dipeptides, namely benzoyl (Bz)-Gly-Arg, Bz-Gly-Phe, carboxybenzoyl (Cbz)-Phe-Ala, Cbz-Glu-Tyr, Cbz-Ala-Phe, Cbz-Phe-Leu, Cbz-Phe-Gly and Cbz-Pro-Phe, by quantification of released amino acids with ninhydrin plus hydrindantin [18]. The activity was determined against 1 mM substrate in the presence of either chelating agents or added metal ions, at different pH values.

For the determination of kinetics parameters, reaction rates were measured under optimal conditions with a substrate concentration range of 0.25–2.0 mM. The values of K_m and V_{max} were determined using a computer-assisted algorithm (Enzfitter; Elsevier-Biosoft, Cambridge, UK).

Endopeptidase activity was determined under the same incubation conditions as in the carboxypeptidase assays by measuring the acid-soluble radioactivity liberated by hydrolysis of ^{125}I -labelled αS1 -casein [8].

PAGE analysis

Analysis of the protein samples throughout the purification of mCP-III was carried out by SDS/PAGE (5–15% gradient or

10% slab gels) according to the Laemmli procedure [19]; the gels were stained with silver nitrate [20].

Peak III from the IMAC column was applied on a 5–20% polyacrylamide Midget gel (LKB) run under non-dissociating conditions. After electrophoresis, the gel was cut into 0.5 cm strips and the proteins were extracted using an electroeluter model 422 (Bio-Rad) for subsequent determination of carboxypeptidase activity.

Protein determination

Protein was assayed by the Bradford method [21], with BSA as the standard. During the course of the chromatography procedures protein was estimated at A_{280} .

RESULTS

Purity of the matrix fraction

When tested by marker enzyme assays, the purified mitochondria contained 1% of the cytosolic marker lactate dehydrogenase and less than 1% of the lysosomal marker acid phosphatase, while the microsomal marker cytochrome *c* reductase was undetectable. The intramitochondrial localization of different enzyme markers has been carefully investigated [8,12,22], demonstrating the purity of the matrix fraction that was used in this study as enzyme source. The matrix fraction contained the bulk of its marker enzyme, malate dehydrogenase, and was practically devoid of contamination by proteins of other mitochondrial fractions, as demonstrated by the absence of succinate dehydrogenase, adenylate kinase and monoamine oxidase activities, which are enzyme markers of, respectively, the inner membrane, intermembrane space and outer membrane fractions.

Separation of carboxypeptidase activities from the matrix extract

The separation of carboxypeptidase activities from the matrix fraction was carried out as described in the Materials and methods section. No carboxypeptidase activity was precipitated up to 30% ammonium sulphate saturation. The protein fraction that was precipitated in the 30–90% saturation range was used as the source for enzyme purification, as it represented a considerable cleaning-up of the matrix extract towards further purification. In addition, due to the high lability of the enzyme, ammonium sulphate precipitate represents the best way of storing the enzymic activity.

The conditions for separation of the matrix proteins by ion-exchange chromatography were determined in a preliminary experiment by using mini-columns of anion or cation exchangers eluted with a stepwise NaCl gradient at different pH values. Carboxypeptidase activities in the eluates were assayed, in the absence or the presence of metal ions, against a number of N-blocked synthetic substrates. The highest recovery of activity was obtained with a DE-52 at pH 7.8 in the presence of Zn^{2+} . By scaling up these conditions and with a linear NaCl gradient four activities could be detected, eluting at 50, 90, 130 and 210 mM NaCl (Figure 1). Each of the four carboxypeptidase activity peaks was incubated with ^{125}I -casein, and only that eluting at 50 mM NaCl revealed endopeptidase activity (results not shown).

The peak with highest carboxypeptidase activity was eluted at 90 mM NaCl (peak II). This enzyme activity also presented the higher stability, and thus it was the one we chose to isolate.

Purification of mCP-III

Fractions corresponding to peak II from the DE-52 column were pooled and further fractionated by f.p.l.c. on a Cu^{2+} -loaded IDA

Figure 1 DEAE ion-exchange chromatography of carboxypeptidase activities from the mitochondrial matrix

A 45 mg sample of the protein fraction that was precipitated at 30–90% ammonium sulphate saturation was desalted in a PD-10 column and applied to a DE-52 column (60 cm × 18 cm) and eluted with a linear NaCl gradient (0–350 mM). The carboxypeptidase activity was eluted in four peaks and was assayed with Bz-Gly-Arg (□) and Cbz-Phe-Ala (▲) in the presence of 25 μM Zn²⁺. Pooled fractions for further purification are indicated by stippling.

Figure 2 IMAC of DE-52 peak II

Fractions corresponding to peak II of carboxypeptidase activity eluted from the DE-52 column were applied to an f.p.l.c. column (Chelating Superose HR 10/2) charged with Cu²⁺. This column was eluted with imidazole up to 20 mM. Carboxypeptidase activity in the eluates was assigned with Bz-Gly-Arg (□) and Cbz-Phe-Ala (▲). Pooled fractions for further purification are indicated by stippling.

column. Four peaks of carboxypeptidase activity against Cbz-Phe-Ala and Bz-Gly-Arg were detected after elution with imidazole up to 20 mM (Figure 2). However, only three of these activities are relevant, inasmuch as the first peak, eluted just at the beginning of the imidazole gradient, showed minimal specific activity. The three remaining carboxypeptidase activities had different substrate specificities against the two N-blocked dipeptides: peaks I and III had preference for Cbz-Phe-Ala, while peak II preferentially hydrolysed Bz-Gly-Arg.

The carboxypeptidase activities were measured directly in the eluates from the IDA–Cu²⁺ column, i.e. in the presence of Cu²⁺. Addition of 1,10-phenanthroline inhibited all three activities.

Figure 3 Gel filtration chromatography of IMAC peak III

Peak III from IMAC (see Figure 2) was concentrated in the presence of 2 mM 1,10-phenanthroline and injected into a Superose 12 HR 10/30 column at a flow rate of 0.3 ml/min. Fractions (600 μl) were tested for carboxypeptidase activity using Cbz-Phe-Ala (▲) and Bz-Gly-Arg (□) as substrates at pH 7.6. Elution positions are shown for molecular mass markers as follows: 1, Blue Dextran (2000 kDa); 2, galactosidase (150 kDa); 3, BSA (66 kDa); 4, chicken albumin (45 kDa); 5, carbonic anhydrase (29 kDa); 6, lactalbumin (14.2 kDa). Fraction 24, corresponding to the mCP-III activity peak, was electrophoresed on a 10% polyacrylamide gel, under denaturing and reduced conditions, and silver stained (inset). Lane 1, molecular mass markers; lane 2, purified mCP-III.

The pooled eluates were made 2 mM in 1,10-phenanthroline to avoid the autolytic inactivation of the carboxypeptidases. If metal ions were added the best re-activation was achieved with Zn²⁺ for peaks I and III (no clear effect was observed for peak II). When these two peaks of activity (I and III) were analysed by SDS/PAGE, peak III consistently showed the highest degree of purity.

F.p.l.c. of IMAC peak III on Superose 12 gave a single major peak of carboxypeptidase activity (Figure 3). Protein was below the detection level of the Bradford method and was therefore estimated by comparing the intensity of the spots on silver-stained gels with protein standards.

mCP-III was purified more than 1700-fold over that in the matrix extract, with a yield of approx. 0.6%. The specific activity of the purified enzyme was about 22000 units/mg of protein. A typical purification is summarized in Table 1.

Molecular mass

The apparent molecular mass of mCP-III was estimated at the last purification step by gel filtration. The enzyme behaved as a ~115 kDa species on Superose 12 gel filtration in the presence of 150 mM NaCl (Figure 3). This final purified preparation of the enzyme was analysed by SDS/PAGE. Silver staining of the gel showed two distinct bands of 61 kDa and 50 kDa (Figure 3, inset), suggesting that the enzyme is a dimer.

The same mCP-III sample was also separated by non-denaturing PAGE and subsequently eluted. The enzyme activity was recovered from a single gel slice, coincident with a faint band of protein (results not shown).

Inactivation studies

When the three carboxypeptidase activities eluted from regular IMAC on a Cu²⁺–IDA gel were depleted of the metal ion by

Table 1 Summary of the purification of mCP-III

About 15 ml of matrix fraction was used as starting material. All purification details are given in the Materials and methods section. One unit of carboxypeptidase activity is defined as the amount of the enzyme that releases 1 μ mol of amino acid per min from Cbz-Phe-Ala. Data represent average values of a number of determinations varying from five to twenty depending on the stage of isolation of the enzyme.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Mitochondria	225	—	—	—	—
Matrix	119.7	1478	0.205	100	1.0
(NH ₄) ₂ SO ₄ (30–90%)	45	1024	0.385	70.5	1.88
DE-52 peak II	1.4	926	11.023	62.6	53.7
IMAC peak III	0.09	624	115.555	42.2	563.7
Superose 12	0.018	391	362.036	26.4	1766

reversed IMAC on a metal-free TED gel, they lost their activities almost entirely.

Inhibition of the three enzymes was also achieved with the metal chelator 1,10-phenanthroline. The mCP-III activity can be clearly distinguished by its inhibition by CPI: pre-incubation of mCP-III with 1 μ g of CPI resulted in more than 60 % inhibition of activity.

Re-activation of the carboxypeptidase apoforms by metal ions

In order to measure the activities of the carboxypeptidase enzymes in the presence of different metal ions, we converted the IMAC-eluted Cu²⁺-containing enzymes to their apoforms by reverse IMAC, carried on a metal-free TED column. Owing to its high affinity for metal ions this column removed the Cu²⁺ from the enzyme, the activity of which could then be subsequently tested in the presence of various metal ions. Table 2 shows the re-activation effect of increasing concentrations of different metal ions on three partially purified carboxypeptidase apoforms obtained by a sequence of direct and reversed IMAC steps. The best re-activation was achieved with Zn²⁺ for peaks I and III (no clear effect was observed on peak II). Co²⁺ mimicked the Zn²⁺ effect. Ca²⁺ activated peak II.

pH optimum, kinetic parameters and enzyme stability

The optimum pH for activity of partially purified mCP-III was measured against Cbz-Phe-Gly, Cbz-Phe-Ala, Cbz-Glu-Tyr, Cbz-Ala-Phe, Bz-Gly-Arg and Bz-Gly-Phe. The pH was varied from 5.0 to 5.5 (0.1 M acetate buffer), from 5.8 to 7.0 (0.1 M sodium phosphate buffer) and from 7.0 to 7.9 (0.1 M Mops). Maximum activity was observed at pH 7.6. The activity fell to low levels below pH 6.5 and above pH 8.0.

The reaction rates with Cbz-Phe-Ala and Bz-Gly-Arg were measured for the purified enzyme at the optimal pH and with substrate concentrations varying between 0.25 and 2.0 mM. The apparent K_m values for the hydrolysis of these two substrates were quite similar, 0.12 ± 0.02 mM for Cbz-Phe-Ala and 0.16 ± 0.05 mM for Bz-Gly-Arg. The V_{max} values were 145.5 ± 4.9 and 86.0 ± 6.5 μ mol/min per mg respectively. In the absence of accurate data on the active enzyme concentrations we were unable to calculate true k_{cat} values, but from the V_{max}/K_m ratios we estimate the catalytic efficiency of the enzyme for Cbz-Phe-Ala to be 2-fold greater than that for Bz-Gly-Arg.

The enzyme is very labile (it loses 90 % of activity overnight at

Table 2 Re-activation of the carboxypeptidase apoform by added metal ions

After reverse IMAC on a metal-free TED column, each carboxypeptidase peak was incubated for 15 min at 30 °C with different concentrations of the metal ion. Assays proceeded by adding the synthetic substrate for which the eluate had a previously determined preference. The restored activity was expressed as a percentage of the original activity.

Addition	Concn. (mM)	Activity (%)		
		Peak I	Peak II	Peak III
Zn ²⁺	0.01	—	0	14
	0.02	23.7	0	30
	0.03	67.1	0	35
	0.04	100.0	0	42
	0.05	80.8	0	100
Co ²⁺	0.025	29.6	0	63
	0.05	71.0	0	107
Cu ²⁺	0.025	13.0	0	45
	0.05	31.0	0	54
Mg ²⁺	0.025	6.0	0	—
	0.05	15.0	0	—
Ca ²⁺	0.1	20.0	0	34
	1	23.0	100	25

Table 3 Substrate specificity of mCP-III

The carboxypeptidase activity of peak III from IMAC was measured under optimal assay conditions. All determinations represent means of at least three separate experiments.

Substrate	Hydrolysing activity (μ mol/min per mg)	Relative rate of hydrolysis
Cbz-Phe-Ala	115.5	1.00
Bz-Gly-Arg	77.4	0.67
Cbz-Phe-Leu	70.4	0.61
Cbz-Phe-Gly	68.1	0.59
Cbz-Ala-Phe	43.9	0.38
Bz-Gly-Phe	31.2	0.27
Cbz-Glu-Tyr	15.0	0.13
Cbz-Pro-Phe	0	0

0 °C if the aliquot is not made 20 % in glycerol) and it is clearly stabilized by Ca²⁺ (10 mM).

Substrate specificity

Table 3 shows the mCP-III activities recorded with a number of N-blocked dipeptides under optimal conditions and at substrate concentrations that were well in excess of the K_m values. The preferential substrate for mCP-III was Cbz-Phe-Ala. Bz-Gly-Arg, Cbz-Phe-Leu and Cbz-Phe-Gly were hydrolysed at appreciable rates, with the others being much less hydrolysed and Cbz-Pro-Phe not hydrolysed at all.

DISCUSSION

This study provides the first demonstration of a carboxypeptidase activity in mitochondria. When searching for a proteolytic activity that is truly mitochondrial in nature, it is essential to isolate mitochondria that are free of contaminants, namely lysosomes and blood components which co-sediment with the mitochondrial fraction. Therefore all our experiments were carried out starting with livers perfused until they were completely

free of blood. Entire lysosomes (containing high concentrations of cysteine proteinases) and generally adsorbed enzymes were efficiently washed off by pre-exposure to digitonin. The subsequently separated mitochondrial fractions have a precise derivation as previously demonstrated in our laboratory [8,12,22]. Thus the carboxypeptidase reported here is indeed localized in the inner core of the organelle, genuinely represented by its matrix fraction.

To prevent proteolysis of the enzymes during the purification procedure, a cocktail of specific inhibitors for serine, cysteine, aspartic and metallo-proteinases was introduced into the isolation medium at the moment of mitoplast rupture. As the agent utilized to inhibit metalloproteinases is the only one of the inhibitors whose action is not irreversible, we were able to detect carboxypeptidase activity by simple addition of metal ions. To ensure that other exopeptidase activities were not interfering with the assays, bestatin (a specific inhibitor of aminopeptidases) was also added to the cocktail of proteinase inhibitors.

Despite these precautions, the mitochondrial carboxypeptidase activities were rapidly inactivated; this was not surprising, since one of the few generalizations that can be made about exopeptidases is that they are more labile than endopeptidases.

For purification of these carboxypeptidase activities we have used IMAC technique. This is a specialized aspect of affinity chromatography based on the co-ordination between electron donor groups (predominantly imidazole and thiol) of a protein surface and transition-metal ions, namely zinc and copper, immobilized on to a chromatographic matrix by an attached chelator. Two fundamentally different types of immobilized metal affinity methods can be applied to proteins: regular IMAC [23] where the metal is present in the adsorbent, and reversed IMAC, where the metal is a structural component of the protein. For separation of proteins regular IMAC is used, where the gel is loaded with the metal through IDA groups and the metal serves as a selective adsorbent for histidine- and cysteine-containing proteins. Reversed IMAC [24] can be used to obtain metal-free forms of a metalloprotein. In this case the metal is attached to the gel by TED groups, which have a stronger retention of metal ions and a weaker retention of proteins relative to the IDA gels. Therefore TED will remove the metal from the protein when this passes through the column.

In the present work we have utilized both types of IMAC. We have performed regular IMAC for separation of carboxypeptidase activities, and reversed IMAC to convert the carboxypeptidase holoenzyme to its apoform, which is not so labile and can be re-activated with metals.

In the light of the observations reported here, it is reasonable to conclude that at least five carboxypeptidase activities can be chromatographically separated from the matrix of rat liver mitochondria. It should be pointed out that our experimental conditions were such that they restricted our investigation to metalloproteinases. Our assumption has been that the carboxypeptidases to be found in the matrix of the rat liver mitochondria should be of the same type as for all animal carboxypeptidases described, i.e. zinc enzymes. However, we cannot exclude the possibility that an organelle such as the mitochondrion might possess enzymes that are evolutionarily different from those of animal cells previously reported.

Three carboxypeptidase activities separated by IMAC could be distinguished on the basis of yield, substrate specificity and inhibition profile. They were all inhibited by the zinc chelator, 1,10-phenanthroline and could be re-activated by the addition of

zinc. The third of these enzymes was the only one that was clearly inhibited by the very specific carboxypeptidase inhibitor CPI. In addition, it was eluted with higher activity and greatest degree of purity. We have therefore pinpointed this third IMAC peak of carboxypeptidase activity as the most interesting of these enzymes and named it mCP-III.

The enzyme has been purified to apparent homogeneity. Its molecular mass is approx. 115000 Da, as judged by gel filtration in non-denaturing conditions. Because two polypeptides, of 61 kDa and 50 kDa respectively, were detected by SDS/PAGE we assumed that the enzyme is active as a dimer.

The enzyme is clearly a carboxypeptidase: it releases the C-terminal residue from N-blocked peptides. The measurable release of a C-terminal alanine is 2-fold faster than that of any other amino acid residue. On the other hand, the rate of hydrolysis seems to be enhanced by the presence of a penultimate phenylalanine residue. In contrast, the enzyme does not act on peptides containing a penultimate proline residue. It is a typical metallo-proteinase, inhibited by the zinc chelator 1,10-phenanthroline and re-activated in a concentration-dependent manner by zinc. Unequivocal demonstration that it is a true metalloenzyme has been obtained with CPI, which binds tightly to carboxypeptidases by direct interaction with the metal at the active site [25].

The novel carboxypeptidase reported here may play an important role in intramitochondrial proteolysis. This is an area of study not yet exploited, although it is of great interest for the complete understanding of the mechanisms underlying the entry, the processing, the modulation and the destruction of proteins within mitochondria.

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