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Rat liver mitochondrial intermediate peptidase (MIP): purification and initial characterization

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A number of nuclearly encoded mitochondrial protein precursors that are transported into the matrix and inner membrane are cleaved in two sequential steps by two distinct matrix peptidases, mitochondrial processing peptidase (MPP) and mitochondrial intermediate peptidase (MIP). We have isolated and purified MIP from rat liver mitochondrial matrix. The enzyme, purified 2250-fold, is a monomer of 75 kDa and cleaves all tested mitochondrial intermediate proteins to their mature forms. About 20% of the final MIP preparation consists of equimolar amounts of two peptides of 47 kDa and 28 kDa, which are apparently the products of a single cleavage of the 75 kDa protein. These peptides are not separable from the 75 kDa protein, nor from each other, under any conditions used in the purification. The peptidase has a broad pH optimum between pH 6.6 and 8.9 and is inactivated by *N*-ethylmaleimide (NEM) and other sulfhydryl group reagents. The processing activity is divalent cation-dependent; it is stimulated by manganese, magnesium or calcium ions and reversibly inhibited by EDTA. Zinc, cobalt and iron strongly inhibit MIP activity. This pattern of cation dependence and inhibition is not clearly consistent with that of any known family of proteases.

Key words: mitochondrial intermediate peptidase (MIP)/ intermediates/mitochondrial processing peptidase (MPP)/ precursor processing/twice-cleaved precursors

Introduction

The vast majority of mitochondrial proteins are encoded in the nucleus and synthesized as larger precursors with amino-terminal leader peptides (presequences) that contain the information required for their targeting and localization to the mitochondria (Rosenberg *et al.*, 1987; Verner and Schatz, 1988; Pfanner and Neupert, 1990). Transport of precursors across the mitochondrial membranes is generally accompanied by proteolytic cleavage of the leader peptides.

Most precursors are cleaved to their mature forms in one step by the mitochondrial processing peptidase (MPP), localized in the mitochondrial matrix. This enzyme has been extensively studied and purified to homogeneity from *Neurospora crassa* (Hawlitschek *et al.*, 1988; Schneider *et al.*, 1990), *Saccharomyces cerevisiae* (Yang *et al.*, 1988), and rat liver mitochondria (Kalousek *et al.*, 1988; Ou *et al.*, 1989; Kleiber *et al.*, 1990; Isaya *et al.*, 1991). In the case of *Neurospora*, the purified enzyme consists of two non-identical proteins, which can be easily separated from each other: a 59 kDa protein with catalytic function and a 52 kDa protein with an enhancing function (Hawlitschek *et al.*, 1988). Such a situation does not seem to apply to MPP isolated from yeast (Yang *et al.*, 1988, 1991) and rat liver mitochondria (Ou *et al.*, 1989; Kleiber *et al.*, 1990; Isaya *et al.*, 1991), where the homologous polypeptides are likely to form a stable heterodimeric complex. With the exception of the 52 kDa protein from rat liver mitochondria, genes for all of these subunits have been cloned and show a high degree of similarity of amino acid sequence (Jensen and Yaffe, 1988; Pollock *et al.*, 1988; Kleiber *et al.*, 1990; Schneider *et al.*, 1990). MPP from all three sources is inhibited by chelators of divalent cations. It is not clear, however, whether the metal ions are required for catalysis or for enzyme conformation, nor is it known which cation is present in the native form of MPP.

A sizeable minority of mitochondrial precursors undergo two-step proteolytic cleavage of the leader peptide by two independent matrix peptidases (Kalousek *et al.*, 1988). These precursors are first cleaved by MPP to give intermediate forms bearing amino-terminal octapeptides that are removed by a second endopeptidase, the mitochondrial intermediate peptidase (MIP), leading to the formation of mature proteins. The octapeptides always contain a large hydrophobic residue like phenylalanine, leucine or isoleucine at position −8 and a small residue, glycine, serine or threonine, at position −5 relative to the amino-terminus of the mature protein (Hendrick *et al.*, 1989; von Heijne *et al.*, 1989; Gavel and von Heijne, 1990). Although two-step processing has long been described for several mitochondrial precursors in *Neurospora* (Hartl *et al.*, 1986; Tropschung *et al.*, 1988), *S.cerevisiae* (Hurt *et al.*, 1985; Fu *et al.*, 1990) and rat liver (Conboy *et al.*, 1982; Sztul *et al.*, 1987, 1988), MIP has not, heretofore, been characterized.

Recently, we reported a partial purification of MIP from rat liver mitochondrial matrix and showed that MIP did not display any proteolytic activity toward precursors that are cleaved twice unless MPP was also present (Isaya *et al.*, 1991), confirming the observation that formation of the intermediate is required for formation of the mature protein (Sztul *et al.*, 1987). On the other hand, this MIP fraction was capable of processing an intermediate form of ornithine transcarbamylase (iOTC), bearing methionine instead of phenylalanine at −8 from the mature amino-terminus (Met-iOTC), to mature OTC without MPP. We have further shown that synthetic octapeptides inhibit the processing activity of partially purified MIP, without affecting MPP activity (Isaya *et al.*, 1992). These data have eliminated the possibility that MIP is a cofactor required by MPP to cleave intermediates into mature proteins and have established that MIP is an independent endopeptidase acting on mitochondrial intermediate proteins as substrates.

We report here the complete purification of MIP from rat liver mitochondrial matrix and our initial characterization of the enzyme and its reactions.

Results

Purification of mitochondrial intermediate peptidase (MIP)

Previous analyses using a variety of mitochondrial precursors revealed the presence of two different processing activities (MPP and MIP) involved in the maturation of nuclearly coded mitochondrial proteins (Kalousek *et al.*, 1988; Isaya *et al.*, 1991). To understand the relationship between MPP and MIP better, we decided to purify MIP from rat liver mitochondrial matrix. We tested the activity of MIP using either Met-iOTC as substrate in the absence of MPP, or pOTC and pMDH (pre-malate dehydrogenase) precursors in the presence of MPP. One unit of activity was defined as the amount of enzyme which processed 50% of the Met-iOTC in 1 μl of a standard translation mixture to the mature form in 10 min at 27°C.

The enzyme was purified from rat liver mitochondrial matrix in five steps, as described in Materials and methods and summarized in Table I. The entire procedure was carried out twice, giving very similar results regarding purity, recovery and specific activity each time. We achieved ~2200-fold purification with a final yield of ~2%. All steps of purification were monitored on SDS–PAGE, and the proteins were stained with Coomassie blue (Figure 1). Attempts to separate MPP from MIP during the first steps of purification were not successful. Although partial separation could be achieved with ω-aminooctyl–agarose, we were able to completely separate MIP from MPP only in the final step of Mono-Q chromatography. The ω-aminooctyl–agarose chromatography was an important intermediate step, however, because it removed a number of proteins which interfered with the Mono-Q separation.

We have already shown that the mature form of OTC generated by the sequential cleavage of pOTC by purified MPP and partially purified MIP has the correct amino-terminus (Isaya *et al.*, 1991). Similarly, cleavage of *in vitro* synthesized Met-iOTC by highly purified MIP resulted in the production of the correct mature OTC amino-terminus (data not shown).

Presence of 47 kDa and 28 kDa peptides in the purified MIP

Our final preparation, when analyzed on SDS–PAGE, consisted of a major polypeptide with an apparent molecular size of 75 kDa (Figure 1, lane 6) and two additional polypeptides of 47 kDa and 28 kDa. The amount of 75 kDa polypeptide was about five times that of the 47 kDa and 28 kDa polypeptides, judged by both Coomassie blue stain and amino acid analysis. The two lower molecular weight polypeptides copurified on BioGel P-200 with the 75 kDa polypeptide, where all protein-containing fractions had the same 5:1:1 ratio of 75 kDa, 47 kDa and 28 kDa polypeptides (not shown). The native molecular size of MIP, estimated from this column, was ~75 kDa (Figure 2). A variety of purification procedures failed to separate these three polypeptides, including isoelectric focusing, where all three components were found to have identical isoelectric points of 5.28, and affinity chromatography on ligands often used for the purification of proteases (data not shown). Therefore, we decided to perform amino acid sequence analysis of amino-terminal and tryptic peptides isolated from all three components. Although the amino-terminus of the 28 kDa peptide appeared to be blocked, we were able to sequence the amino-termini of the 75 kDa and 47 kDa proteins and found them to be identical (Figure 3A). In addition, the sequence of one large peptide from the 28 kDa protein precisely matched those of two peptides from the 75 kDa species (Figure 3B). These findings indicate that the 47 kDa and 28 kDa polypeptides are the products of cleavage of the 75 kDa protein, probably at a unique position. Such a cleavage is not sufficient to cause separation of the 47 kDa and 28 kDa polypeptides from each other during MIP purification. We do not know when this cleavage occurs or what protease is responsible, but our preliminary data indicate that it is not a self-catalytic process, because incubation of purified MIP under the conditions described in Materials and methods does not change the ratio of 75 kDa, 47 kDa and 28 kDa proteins. Similarly, proteolysis of MIP by trypsin or proteinase K generated much smaller proteolytic products than the 47 kDa and 28 kDa polypeptides (data not shown).

Properties of the purified MIP

Divalent cation requirement of MIP. When we tested MIP activity with *in vitro* translated precursor in the presence of MPP or with Met-iOTC in the absence of MPP, we always observed cleavage into the corresponding mature form. To test for a divalent cation requirement, we dialyzed the *in vitro* translated substrates using Centricon 30 to remove Mg^{2+} and other cations which are present in the reticulocyte lysate. When dialyzed Met-iOTC was incubated with MIP without any additions, we were still able to detect small amounts of conversion to the mature form (Figure 4A,

Table I. Purification of MIP from rat liver mitochondria

Fraction	Total protein (mg)	Total activity ($U \times 10^{-3}$)	Specific activity (U/mg)	Purification factor	Yield (%)
Matrix	15 750	210	13.3	1.0	100
DEAE Bio-Gel A	1395	159	114	8.5	76
Heparin–agarose	217	72	331	24.8	34
Hydroxyapatite	28.7	54	1881	141	25
ω-Aminooctyl–agarose	1.3	9.4	7230	543	4.5
Mono-Q	0.14	4.4	29 950	2251	2.1

One unit of activity is defined as the amount of enzyme that catalyzes the conversion of 50% of the Met-iOTC in 1 μl of *in vitro* translation mixture to its mature form in 10 min at 27°C.

lane 1). Addition of 1 mM Mn^{2+}, Mg^{2+} or Ca^{2+} stimulated the activity 4.5-, 4.8- and 2.2-fold, respectively (Figure 4A, lanes 2–4), while 1 mM Co^{2+}, Fe^{2+} or Zn^{2+} inhibited MIP activity completely (compare Figure 4A, lane 1 with lanes 5–7). At a concentration of 0.1 mM, some degree of stimulation by Mn^{2+}, Mg^{2+} and Ca^{2+} was still observed, and Zn^{2+} and Co^{2+} were still strong inhibitors; Fe^{2+} was without effect at this concentration (data not shown). When we performed the processing reaction in the presence of both 0.1 mM Mn^{2+} and 0.1 mM Zn^{2+} [standard conditions in most of our previous studies when using non-dialyzed substrates (Isaya *et al.*, 1991)], the inhibitory effect of Zn^{2+} was much lower (29% inhibition) (Figure 4B). To exclude the possibility that these effects were specific only for the conversion of Met-iOTC to OTC, we used another substrate, pMDH, which is also processed in two sequential steps (Sztul *et al.*, 1988). When we incubated dialyzed pMDH in the presence of purified MPP and MIP, the first reaction, i.e. conversion of the precursor into the intermediate, was slightly inhibited by Co^{2+} (12%) and only partially inhibited by Zn^{2+} (54%), whereas the second reaction, i.e. conversion of iMDH to mature MDH, was fully inhibited by these two metal ions (Figure 4C). The

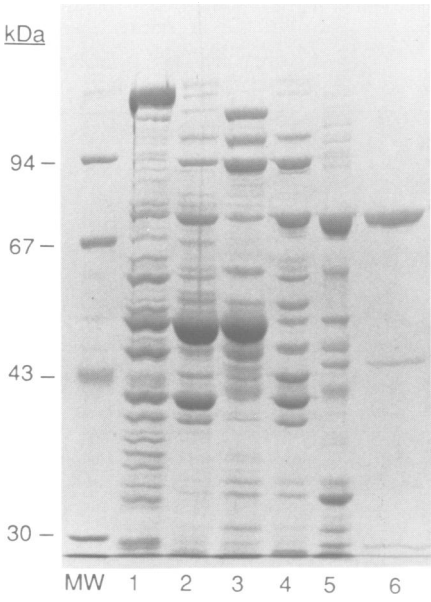

Fig. 1. Purification of MIP as analyzed by SDS–PAGE. 15 μl aliquots of different fractions were electrophoresed on 7% SDS–PAGE. Proteins were stained with Coomassie brilliant blue R-250. A Pharmacia calibration kit was used for protein standards (lane MW). Lane 1, mitochondrial matrix (60 μg); lane 2, DEAE Bio-Gel pool (50 μg); lane 3, heparin–agarose eluate (40 μg); lane 4, hydroxyapatite pool (25 μg); lane 5, ω-aminooctyl–agarose pool (12 μg); lane 6, Mono-Q pool (2.5 μg).

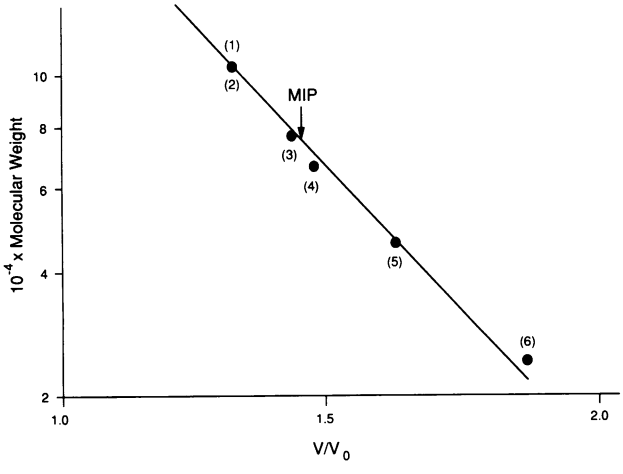

Fig. 2. Estimation of the molecular size of MIP on Bio-Gel P-200. Calibration proteins were: 1, OTC (108 kDa); 2, mitochondrial processing peptidase (MPP) (107 kDa); 3, arginyl-tRNA synthetase (78 kDa); 4, bovine serum albumin (67 kDa); 5, ovalbumin (43 kDa); and 6, chymotrypsinogen (25 kDa).

A1 Val - Ser - Thr - Ser - X - X -Pro - X - Gly - Ala - X - Phe - Asn

A2 Val - Ser - Thr - Ser - X - X- Pro - X - Gly -Ala - X - Phe - Asn - Val - Lys - Pro - Gln

B1 Glu - Tyr - Phe - Ser - Asn - Asp - Tyr - Arg

B2 X - X - Ser - Gln - Phe - Ala - Lys

B3 Glu - Tyr - Phe - Ser - Asn - Asp - Tyr - X - Val - Val - Ser - Gln - Phe - Ala - Lys

Fig. 3. Amino acid sequences of the amino-termini of 75 kDa (A1) and 47 kDa (A2) polypeptides and of tryptic peptides isolated from 75 kDa (B1 and B2) and 28 kDa (B3) polypeptides. The identical sequences are underlined.

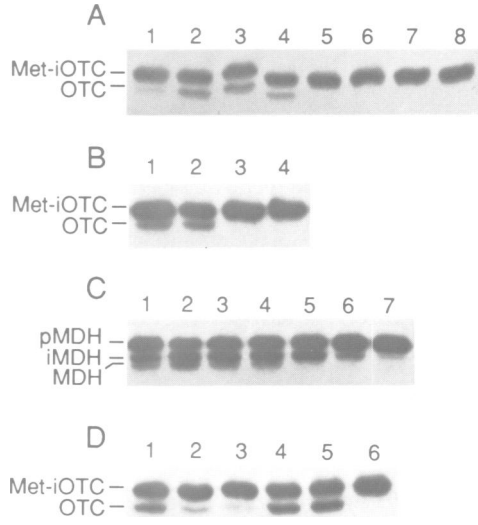

Fig. 4. Metal ion requirement for MIP processing activity. **A.** Aliquots of a Met-iOTC translation mixture, dialyzed on a Centricon 10, were incubated with purified MIP in the absence (lane 1) or presence of 1 mM Mn^{2+} (lane 2), Mg^{2+} (lane 3), Ca^{2+} (lane 4), Co^{2+} (lane 5), Zn^{2+} (lane 6) or Fe^{2+} (lane 7) for 15 min at 27°C. Lane 8, translation only. The products were analyzed directly by SDS–PAGE and fluorography. **B.** Non-dialyzed Met-iOTC translation mixture was incubated with MIP in the presence of either 0.1 mM Mn^{2+} (lane 1), 0.1 mM each Mn^{2+} and Zn^{2+} (lane 2) or 0.1 mM Zn^{2+} (lane 3) alone under the same conditions as in panel A. Lane 4, translation only. **C.** Aliquots of dialyzed pMDH translation mixture were incubated with purified MPP and MIP in the absence (lane 1) and presence of 1 mM Mn^{2+} (lane 2), Mg^{2+} (lane 3), Ca^{2+} (lane 4), Co^{2+} (lane 5) or Zn^{2+} (lane 6) for 15 min at 27°C. Lane 7, translation only. **D.** Purified MIP was incubated in the absence (lane 1) or presence of either 0.01 mM (lanes 2 and 4) or 0.05 mM (lanes 3 and 5) EDTA for 10 min at 27°C. Dialyzed Met-iOTC translation mixture was then added to each sample and the Mn^{2+} concentration brought to 1 mM for lanes 1, 4 and 5. Lane 6, translation only. Samples were incubated for an additional 15 min at 27°C and analyzed as described in Figure 4.

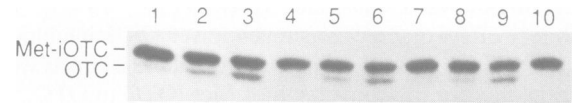

Fig. 5. Treatment of the translation mixture with metal ions. Dialyzed Met-iOTC translation mixture was incubated with 1 mM Mn^{2+} (lanes 1–3), Co^{2+} (lanes 4–6) or Zn^{2+} (lanes 7–9) for 10 min at 27°C. The metals were then removed by Centricon 30 dialysis and the translation mixtures were incubated with purified MIP in the absence (lanes 1, 4 and 7) or presence of 0.1 mM (lanes 2, 5 and 8) or 1 mM (lanes 3, 6 and 9) Mn^{2+} for 15 min at 27°C. Lane 10, translation only. The products were analyzed as in Figure 4.

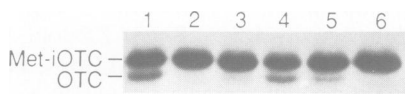

Figure 6. Effect of sulfhydryl group inhibitors on MIP processing activity. Purified MIP was incubated in the absence (lane 1) or presence of 0.2 mM NEM (lane 2), *p*-hydroxymercuribenzoate (lane 3), iodoacetic acid (lane 4) or iodoacetamide (lane 5) for 15 min at 27°C. Met-iOTC translation was added and the incubation continued for an additional 15 min at 27°C. Lane 6, translation only. The products were analyzed as in Figure 4.

presence of Mn^{2+} was still necessary for the optimal processing of iMDH, increasing the formation of MDH by 95%.

We have already reported the effect of EDTA on partially purified MIP using *in vitro*-translated, non-dialyzed precursor as a substrate for two-step conversion by MPP and MIP (Kalousek *et al.*, 1988). To verify that the second reaction, i.e. conversion of the intermediate into the mature form by MIP, is chelator-sensitive, we incubated dialyzed Met-iOTC with MIP in the presence of EDTA. Addition of EDTA at a final concentration of 0.01 mM inhibited the processing activity by 69% (Figure 4D, lane 2), whereas 0.05 mM EDTA resulted in inhibition of 85% (Figure 4D, lane 3). When the EDTA-inhibited MIP was treated with either 0.1 mM or 1 mM Mn^{2+}, the activity was fully restored (Figure 4D, lanes 4 and 5).

To exclude the possibility that Co^{2+} and Zn^{2+} affect substrate processibility rather than the activity of MIP itself, we preincubated Met-iOTC with 1 mM Mn^{2+}, Co^{2+} or Zn^{2+}. After removing the metals by Centricon 30 dialysis, the translation mixture was incubated with MIP without additional Mn^{2+}. As shown in Figure 5, pre-treatment of the translation mixture with any of the three metals, followed by dialysis, resulted in reduced processing of iOTC to mature OTC. Addition of Mn^{2+} restored the processing to the normal level. Even when the translation mixtures pre-treated with Co^{2+} or Zn^{2+} were used, processing was restored to 39 and 23% by 0.1 mM Mn^{2+} (lanes 5 and 8) and to 48 and 49% by 1 mM Mn^{2+} (lanes 6 and 9).

Inhibition of MIP by sulfhydryl group inhibitors

We have already reported (Kalousek *et al.*, 1988) that MIP activity, but not MPP activity, can be inhibited by incubation of crude mitochondrial matrix with *N*-ethylmaleimide (NEM). We extended these studies using purified MIP and other sulfhydryl group inhibitors. As shown in Figure 6 (lane 2), when pure MIP is incubated with NEM at a final concentration of 0.2 mM, the conversion of iOTC to OTC is fully inhibited. Marked, but not full, inhibition was also seen with 0.2 mM *p*-hydroxymercuribenzoate (Figure 6, lane 3). Iodoacetamide and iodoacetic acid at 0.2 mM had much smaller effects on the activity of MIP, with inhibition of 63% and 44%, respectively (Figure 6, lanes 4 and 5). Inhibition by these reagents suggests that the sulfhydryl group of one or more cysteine residues is required for MIP activity.

Other properties of MIP

To address the question of whether MIP can be included as a member of one of the known classes of proteases, we tested several known class-specific protease inhibitors. Many of them inhibited the activity of MIP (see Table II), and the pattern suggested that MIP may belong to the group of cysteine proteases which are often inhibited by leupeptin, N^{α}-tosyl-lysyl chloromethyl ketone (TLCK) and N^{α}-tosyl-phenylalanyl-chloromethyl ketone (TPCK), as well as by sulfhydryl-group inhibitors like NEM. On the other hand, this group of proteases is not usually affected by divalent cation chelators. MIP was also inhibited by N^{α}-*p*-tosyl-L-arginine methyl ester (TAME), which is a substrate for plasmin (Sodetz and Castellino, 1972). Using standard conditions for cleavage of TAME by plasmin, we were not able to show that this compound served as a substrate for MIP (data not shown).

Table II. Inhibition of MIP

Inhibitor	Concentration (mM)	Inhibition (%)
Leupeptin	0.1	60
	1.0	100
Pepstatin	0.1	100
TPCK	0.1	100
TLCK	0.1	70
	1.0	100
Bestatin	0.1	100
Amastatin	1.0	60
PMSF	1.0	0
Benzamidine	1.0	0
TAME	1.0	85

Purified MIP was incubated with Met-iOTC translation mixture for 15 min at 27°C with the indicated concentration of inhibitors. Translation products were analyzed by scanning densitometry of fluorographs.

Purified MIP has a broad pH optimum (between pH 6.6 and 8.8, using Tris or HEPES as buffers). It is not inhibited by either NaCl or KCl at concentrations up to 200 mM and is stable at −70°C for at least 6 months.

Discussion

We have previously shown that the maturation of a number of mitochondrial proteins requires two distinct peptidases, the mitochondrial processing peptidase (MPP) and the mitochondrial intermediate peptidase (MIP). These twice-cleaved precursors are first cleaved by MPP, leading to the formation of intermediates with specific amino-terminal octapeptides. The mature protein is then generated by cleavage of the octapeptide by the second endopeptidase, MIP (Kalousek *et al.*, 1988). We have also shown that, in twice-cleaved precursors, deletion of the octapeptide results in no cleavage by either peptidase. In addition, no cleavage

occurs when the leader peptide of a once-cleaved precursor is joined directly to the mature amino-terminus of a twice-cleaved precursor (Isaya *et al.*, 1991). From these results, we concluded that the mature amino-terminus of twice-cleaved precursors is incompatible with cleavage by MPP and that these proteins require octapeptides cleaved by MIP to overcome this incompatibility (Isaya *et al.*, 1991). Our recent results revealed that synthetic octapeptides can inhibit the processing activity of partially purified rat liver MIP and that the amino-terminal hydrophobic residue in the octapeptides plays a significant role for recognition of the intermediates by MIP (Isaya *et al.*, 1992).

While MPP has been purified from *Neurospora*, yeast and rat liver mitochondria, MIP has, until now, been only partially purified and characterized from rat liver mitochondria. Its presence has also been recently postulated in yeast mitochondria, where the precursor of the Rieske iron–sulfur protein is processed into the mature form in two sequential steps (Fu *et al.*, 1990). Attempts to isolate and further characterize this enzyme from yeast have been unsuccessful thus far (Badran and Beattie, 1992).

In the present study, we have purified MIP from rat liver mitochondrial matrix. The enzyme preparation contains a major 75 kDa peptide and two additional 47 kDa and 28 kDa peptides, which are the products of a single cleavage in the 75 kDa protein by an unknown protease. Which of the proteins, the single 75 kDa protein or the tightly bound complex of 47 kDa and 28 kDa peptides, or both, represents the active enzyme? Because we have not been able either to prevent the partial proteolysis which may occur during the purification, or to achieve conversion of 75 kDa to the smaller polypeptides, or to separate the 75 kDa protein from the cleaved material, we cannot answer this question at the present time. Because of the small amounts of MIP recoverable from rat liver mitochondria, this answer must await cDNA cloning and expression of the cloned protease under conditions which would prevent proteolysis. Such material might also allow us to identify and characterize the protease responsible for the cleavage of the 75 kDa protein.

The rather low recovery of MIP can be partially explained by the lability of the enzyme during the first three steps of purification. The major reason for the low recovery, however, was the fact that we were forced to combine only fractions with the highest MIP activity after heparin–agarose and ω-aminooctyl–agarose chromatography, because of the substantial overlap of MIP activity with MPP and other proteins which we were not able to separate from MIP in subsequent steps.

There exists a considerable amount of data regarding cation requirements for MPP, permitting us to compare MPP and MIP in this regard. MPP from *Neurospora* requires Mn^{2+}, which can be substituted for by Zn^{2+} or Co^{2+} (Schneider *et al.*, 1990). Similarly, MPP activity in yeast can be reactivated with either Zn^{2+}, Co^{2+} or Mn^{2+} after inhibition by chelator (Yang *et al.*, 1991). In our laboratory, we have routinely used a combination of 0.1 mM Zn^{2+} and 0.1 mM Mn^{2+} in the processing reaction. The effect of this mixture on the processing of precursors is very similar to that of Mn^{2+} alone, while Zn^{2+} alone at this concentration does not stimulate and higher concentrations inhibit processing (F.Kalousek, unpublished). These results are in agreement with the studies of Kumamoto *et al.* (1986), who found inhibitory effects of Zn^{2+} on matrix proteases from two mammalian tissues, bovine adrenal cortex and rat liver. Both exhibited full activity in the presence of either Mn^{2+} or Co^{2+}.

Purified MIP also requires manganese ions for full activity, with Mg^{2+} and Ca^{2+} having a stimulatory effect on the peptidase when a dialyzed translation mixture is used in the processing reaction. Not only Zn^{2+}, but also Co^{2+} and Fe^{2+} were found to be strong inhibitors of MIP. Inhibition by low concentrations of Zn^{2+} could be partially offset by the presence of at least an equivalent amount of Mn^{2+}. These results are in disagreement with our previously published observations (Conboy *et al.*, 1982) in which Zn^{2+}, Co^{2+} or Mn^{2+}, but not Mg^{2+} or Ca^{2+}, were required for the conversion of *in vitro*-translated pOTC to the mature form by crude rat liver mitochondrial matrix. The reason for this discrepancy is not clear. It is possible, for example, that MIP loses its endogenous metal ion co-factor during purification without irreversible inactivation of the enzyme, as has been recently postulated for yeast MPP (Yang *et al.*, 1991). The endogenous metal species might not be displaced in crude matrix by Zn^{2+} or Co^{2+}, but, once removed during purification, it can be replaced by Zn^{2+} or Co^{2+} to yield an inactive enzyme. The reversal of the inhibition by equimolar or higher concentrations of Mn^{2+} suggests a relatively low affinity for the inhibitor metal ions. In summary, both peptidases involved in the maturation of mitochondrial precursors require metal ions for their activity but, from present evidence, it is not clear which are bound to the native enzyme *in vivo* and function in the mitochondrial matrix.

A major difference between rat liver MPP and MIP is in their sensitivity to the sulfhydryl reagent, NEM; it fully inhibits MIP processing activity, but has no effect on MPP (Kalousek *et al.*, 1988). This insensitivity of MPP toward NEM does not apply, however, to all MPP homologs; *Neurospora* MPP is nearly fully inhibited by NEM (Schneider *et al.*, 1990), although at higher concentrations. We will be better able to assess the involvement of specific cysteine residues in the activity of MIP when we have information about the primary structure of the enzyme. Because the inhibition and cation dependence patterns described above are not consistent with those of any of the known families of proteases, such structural data may also allow us to determine whether MIP is a member of one of these groups of related proteins.

Even though a considerable amount of data has been gathered about the structural requirements for cleavage of the intermediates by MIP (Sztul *et al.*, 1987; Hendrick *et al.*, 1989; von Heijne *et al.*, 1989; Isaya *et al.*, 1991, 1992), its catalytic mechanism is still not known. It is likely that this peptidase requires a hydrophobic residue at the amino-terminus of the octapeptide and some higher order structure around the cleavage site, similar to what has been described for 'magaininase', another metalloprotease which requires a specific amphiphatic α-helical motif composed of at least 12 residues for the processing of magainin peptides of *Xenopus* (Resnick *et al.*, 1991).

Only two matrix peptidases, MPP and MIP, which are involved in the processing of mitochondrial proteins have been characterized so far (Schneider *et al.*, 1991). A third, highly specialized peptidase resides in the inter-membrane space. We cannot exclude the possibility that other peptidases are involved in the processing of certain mitochondrial

precursors. For example, when human OTC was expressed in *Spodopter frugiperda* (*Sf*) insect cells using a baculovirus vector, three forms of the enzyme accumulated—iOTC, mature OTC, and a novel intermediate which contained only the four carboxyl-terminal residues of the leader peptide (Lightow *et al.*, 1991). The latter protein is probably a product of cleavage by another protease, because it is not observed using purified MPP or MIP. Whether this truly represents the action of another enzyme or simply a relaxed specificity of one of the known proteases must await further dissection of the insect cell system.

Materials and methods

Miscellaneous sources and methods

DEAE Bio-Gel A was from Bio-Rad Laboratories (Richmond, CA). The Mono-Q anion exchange column was purchased from Pharmacia Chemicals (Piscataway, NJ). Hydroxyapatite was prepared by the method of Main *et al.* (1959) using ammonia for neutralization at boiling temperature. Heparin–agarose and ω-aminooctyl–agarose were from Sigma Chemical Co. (St Louis, MO). Centriprep 30 and Centricon 30 concentrators were from Amicon Co. (Beverly, CA). Rabbit reticulocyte lysate was from Promega Corp. (Madison, WI). SDS–PAGE was carried out as described by Laemmli (1970) with either of two different ratios of acrylamide:*bis*-acrylamide (23:1 or 33:1).

In vitro transcription, translation and processing

Messenger RNAs were prepared by *in vitro* transcription from various wild-type cDNAs cloned in transcription vectors. Transcription with either phage SP6 or phage T7 polymerase (Boehringer) and *in vitro* translation using rabbit reticulocyte lysate (Promega Corp.) and [^{35}S]methionine (Amersham Corp.) were performed according to their suppliers' recommendations. Processing of *in vitro* translated mitochondrial precursors with mitochondrial processing peptidases was, unless otherwise stated, carried out at 27°C for 10 min. In a typical experiment, the enzyme preparation was diluted to the required concentration with 10 mM HEPES, pH 7.4, 1 mM DTT, and 0.1 mg/ml bovine serum albumin and incubated with 1 μl of translation mixture in a total volume of 10 μl with 1 mM (final concentration) Mn^{2+}. Products of the reaction were analyzed on SDS–PAGE, detected by fluorography and quantified by densitometry of fluorograms using a BioImage Visage 200 densitometer.

Protein and peptide sequencing

To avoid contamination, all analyzed proteins were first separated on SDS–PAGE and transferred to Immobilon-P membrane (Millipore Corp.) by electroblotting, visualized by Coomassie blue, cut out from the membrane and sequenced using an Applied Biosystems model 470A or 477 sequenator (Matsudaira, 1987). For the generation of tryptic peptides, ~100 pmol of protein was electrophoresed and blotted as described above. CNBr and tryptic cleavage, elution and separation of peptides, and their amino acid sequencing, were done as described by Stone *et al.* (1990). Radiosequencing of mature OTC formed upon cleavage of Met-iOTC by purified MIP was performed as described previously (Isaya *et al.*, 1991).

Preparation of mitochondrial matrix

All operations were carried out at 0–5°C unless otherwise stated. Usually, 20 male Sprague-Dawley rats between 90–130 g were used at one time. Intact liver mitochondria were prepared as described previously (Conboy *et al.*, 1982). Mitochondria were separated into outer membrane, intermembrane space and mitoplast fractions by additional treatment with digitonin (Schnaitman and Greenawalt, 1968). Matrix was prepared by treatment of mitoplasts with Lubrol WX (Schnaitman and Greenawalt, 1968). For the preparation of MIP, we pooled matrix fractions from two batches of rats processed sequentially (see below).

Purification of mitochondrial intermediate peptidase (MIP)

General remarks. The first three steps of purification were usually carried out using mitochondrial matrix prepared from the liver of 40 rats. Attempts to scale up these three steps were not successful: a lower yield resulted because of the greater time required. The enzyme was rapidly inactivated both in matrix (~50% after 24 h at 4°C) and after the first step of purification, requiring us to carry out the first three steps of purification within 72 h.

DEAE Bio-Gel A chromatography. The mitochondrial matrix fraction (~2000 mg) was diluted with 10 mM HEPES, pH 7.4, 1 mM DTT, brought to 20 mM NaCl in a final volume of 400 ml, and loaded on a DEAE Bio-Gel A column (2.5×9 cm), equilibrated with the same buffer, at a flow rate of 120 ml/h. After adsorption, the column was washed with two column volumes of the same buffer, followed by washing with 16 column volumes of 30 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM DTT. The peptidase was then eluted with a 500 ml linear gradient from 30 mM NaCl to 150 mM NaCl in 10 mM HEPES, pH 7.4, 1 mM DTT, at a flow rate of 60 ml/h. Fractions with a NaCl concentration between 70 mM and 100 mM were pooled, concentrated on a Centriprep 30 concentrator to 20 ml, diluted to 100 ml with 10 mM HEPES, pH 7.4, 1 mM DTT, and reconcentrated to 25 ml. The concentration of NaCl was then adjusted to 20 mM.

Heparin–agarose chromatography. The concentrated sample from the previous step was loaded on a heparin–agarose column (2.5×6 cm), equilibrated with 20 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM DTT, at a flow rate of 24 ml/h. The column was eluted with the same buffer at the same flow rate. The first 50 ml of the eluate were used in the subsequent step.

Hydroxyapatite chromatography. The heparin eluate was diluted with 10 mM HEPES, pH 7.4, 1 mM DTT, to 100 ml and applied to a hydroxyapatite column (1.5×4.6 cm), equilibrated with 10 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM DTT, at a flow rate of 24 ml/h. After adsorption, the column was washed extensively with 10 mM potassium phosphate, pH 7.0, 1 mM DTT. About 40% of the total protein and <10% of MIP eluted and was discarded. The major portion of MIP activity was eluted by a 150 ml linear gradient from 10 mM potassium phosphate, pH 7.0, 1 mM DTT, to 60 mM potassium phosphate, pH 7.6, 1 mM DTT (3.5 ml fractions). Proteins from every other fraction were analyzed on SDS–PAGE using gels with both 23:1 and 33:1 acrylamide:*bis*-acrylamide ratios. Using the 23:1 ratio, we were able to distinguish MIP from a protein which has a slightly faster mobility and which starts to be eluted earlier than MIP. Using a 33:1 ratio, we detected a more slowly moving impurity which is present in at least 10-fold excess over MIP protein and which had the same mobility as MIP when the ratio was 23:1. Only the fractions which were virtually free of both of these two contaminants were pooled (usually between 26 and 42 mM potassium phosphate), concentrated to 5 ml on a Centriprep 10 concentrator, frozen on dry ice and kept at 70°C. These three steps were then repeated until the matrix fractions from 300–320 rats had been processed to this point.

ω-Aminooctyl–agarose. Pools from eight hydroxyapatite columns were combined and diluted with 50 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM DTT, to 100 ml and loaded on a 1.5 × 6 cm ω-aminooctyl-agarose column, equilibrated with the same buffer, at a flow rate of 24 ml/h. The column was washed with 12 column vol of 150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM DTT. The MIP activity was eluted with a 500 ml linear gradient from 150 mM to 500 mM NaCl in 10 mM HEPES, pH 7.4, 1 mM DTT, at a flow rate of 24 ml/h. The fractions were analyzed for protein on SDS–PAGE and assayed for enzyme activity. The active fractions, free of most MPP activity (300–340 mM NaCl), were pooled, concentrated, diluted with 10 mM HEPES, pH 7.4, 1 mM DTT, and reconcentrated on a Centriprep 10 to a final NaCl concentration of 20 mM and a final volume of 3 ml.

FPLC Mono-Q chromatography

Half of the pool from the previous step was loaded on a Mono-Q anion-exchange column (HR 5/5) which had been equilibrated with the same buffer. After washing the column with 2 ml of the same buffer and 6 ml of 120 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM DTT, MIP activity was eluted with a 30 ml linear gradient between 120 mM and 280 mM NaCl in 10 mM HEPES, pH 7.4, 1 mM DTT. The MIP activity eluted between 165 and 205 mM NaCl. The active fractions were pooled, concentrated, diluted with 10 mM HEPES, pH 7.4, 1 mM DTT, reconcentrated with a Centricon 10 to a final volume of 300 μl (20 mM NaCl) and stored at −70°C.

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