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Purification and characterization of a processing protease from rat liver mitochondria

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A processing protease has been purified from the matrix fraction of rat liver mitochondria. The purified protease contained two protein subunits of 55 kd (P-55) and 52 kd (P-52) as determined by SDS–PAGE. The processing protease was estimated to be 105 kd in gel filtration, indicating that the two protein subunits form a heterodimeric complex. At high ionic conditions, the two subunits dissociated. The purified processing protease cleaved several mitochondrial protein precursors destined to different mitochondrial compartments, including adrenodoxin, malate dehydrogenase, P-450(SCC) and P-450(11β), but the processing efficiencies were different each other. The endoprotease nature of the processing protease was confirmed with the purified enzyme using adrenodoxin precursor as the substrate; both the mature form and the extension peptide were detected after the processing. The processing activity of the protease was inhibited by metal chelators, and reactivated by Mn^{2+}, indicating that the protease is a metalloprotease.

Key words: processing protease/endoprotease/purification/rat liver mitochondria/protein import

Introduction

The majority of mitochondrial proteins are encoded by the nuclear genome and cytoplasmically synthesized as larger precursors (Neupert and Schatz, 1981; Hay *et al.*, 1984). The precursors carry cleavable extension peptides at their amino termini, which function as the targeting signal to direct the precursors to mitochondria (Hurt *et al.*, 1984; Horwich *et al.*, 1985). *In vitro* and *in vivo* studies have demonstrated that the precursors are post-translationally imported into mitochondria by a multi-step process that involves unfolding of the precursors (Eilers and Schatz, 1988), specific binding of the precursors to the mitochondrial surface (Hennig and Neupert, 1981; Zwizinski *et al.*, 1983; Ono and Ito, 1984b) and energy-dependent translocation of the precursors across the mitochondrial membranes (Nelson and Schatz, 1979; Gasser *et al.*, 1982; Scheleyer *et al.*, 1982). In most cases, the import of the precursors into mitochondria is accompanied by removal of the extension peptides from the precursors (Maccecchini *et al.*, 1979; Nabi and Omura, 1980; Mihara *et al.*, 1982; Teintze *et al.*, 1982). The proteolytic processing of the precursors is catalyzed by a processing protease located in the mitochondria (Conboy *et al.*, 1982; McAda and Douglas, 1982; Bohni *et al.*, 1983). Processing protease has high specificity for mitochondrial protein precursors, and correctly cleaves the extension peptides from the precursors (Bohni *et al.*, 1983; Cerletti *et al.*, 1983; Schmidt *et al.*, 1984). Although the processing of the precursors was independent of their translocation into the mitochondria (Zwizinski and Neupert, 1983; Ou *et al.*, 1986), removal of the extension peptides by processing protease has been suggested to be required for assembly into active enzymes (Kalousek *et al.*, 1984; Isaya *et al.*, 1988).

It has been reported that different proteases are involved in the processing activity (Ogishima *et al.*, 1985; Kalousek *et al.*, 1988). Some precursors, such as cytochrome c_1, cytochrome b_2, and the Fe–S protein of complex III, are cleaved by two steps, and the cleavages at different sites have been suggested to be catalyzed by different enzymes (Ohashi *et al.*, 1982; Daum *et al.*, 1982; Hartl *et al.*, 1986). Processing protease located in mitochondrial matrix from different sources have been characterized (McAda and Douglas, 1982; Miura *et al.*, 1982; Bohni *et al.*, 1983; Sagara *et al.*, 1984; Schmidt *et al.*, 1984). The enzyme cleaves most mitochondrial protein precursors, and differs from the signal peptidases of endoplasmic reticulum and bacteria in that it is a metalloprotease (Zwizinski and Wickner, 1980; Evans *et al.*, 1986). The matrix processing protease was purified from *Neurospora crassa* (Hawlitschek *et al.*, 1988). Two different proteins, namely mitochondrial processing peptidase (MPP) and processing enhancing protein (PEP) were required for the full processing activity. Since the two proteins were separated from each other by gel filtration they did not form a complex. Two yeast mutants that accumulated uncleaved mitochondrial protein precursors at the restrictive temperature were isolated (Yaffe and Schatz, 1985; Yaffe *et al.*, 1985). Analysis of the two mutants revealed that the mutations are related to the two protein components of the mitochondrial processing protease (Jensen and Yaffe, 1988; Pollock *et al.*, 1988; Witte *et al.*, 1988), indicating that similar enzymes are involved in the processing of mitochondrial protein precursors in fungi and yeast.

Although purification of the matrix processing protease has been attempted with rat liver and bovine adrenal cortex mitochondria (Miura *et al.*, 1982; Sagara *et al.*, 1984; Kumamoto *et al.*, 1986), purified preparation has never been obtained from mammalian sources. In this study we report the purification of a matrix processing protease to homogeneity from rat liver mitochondria using adrenodoxin precursor as the substrate. The purified processing protease contained two protein components of 55 and 52 kd, suggesting a dimeric structure of the processing protease. Judging from the processing of adrenodoxin precursor, the purified protease was an endoprotease, and its activity was dependent on divalent metals.

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Table I. Purification of processing protease from rat liver mitochondria

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Mt	45 200	–	–	–	–
Matrix	15 600	206 000	10.5	1.0	100.0
DEAE	2 300	157 000	68.0	6.5	75.9
C_6–Seph	294	98 200	334	31.8	47.6
G-150	18.9	56 000	2 980	283	27.3
HA	3.5	34 000	9 700	922	16.4
Mono-Q I	1.1	21 600	18 700	1780	10.0
Mono-Q II	0.54	13 500	25 100	2390	6.6

One unit of the processing activity is defined as the amount of the enzyme that cleaves 50% of adrenodoxin precursor to the mature form under the assay condition described in Materials and methods.

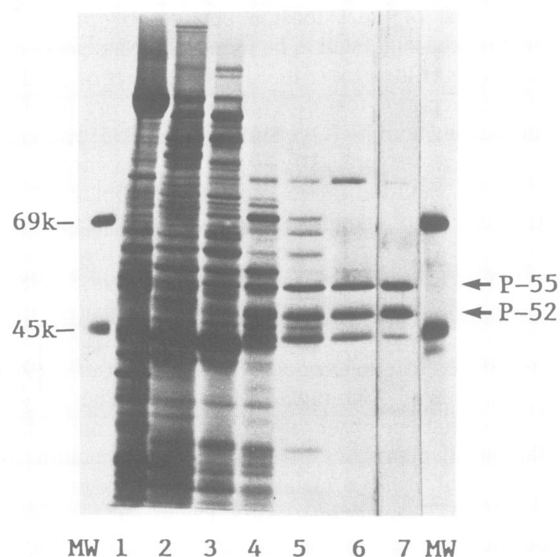

Fig. 1. SDS–PAGE at purification steps. Samples of different purification steps were subjected to SDS–PAGE using 8% polyacrylamide gel followed by silver staining. BSA (69 kd) and ovalbumin (45 kd) were used as the mol. wt markers (MW). P-55 and P-52 represent the two subunits of the processing protease. **Lane 1**, matrix fraction (30 μg); **lane 2**, DEAE–cellulose fraction (6 μg); **lane 3**, aminohexyl–Sepharose 4B fraction (1.5 μg); **lane 4**, Sephadex G-150 fraction (600 ng); **lane 5**, hydroxylapatite fraction (300 ng); **lane 6**, FPLC Mono-Q I fraction (150 ng); **lane 7**, FPLC Mono-Q II fraction (150 ng).

Results

Purification of processing protease

The matrix fraction of rat liver mitochondria contains a processing activity that processes adrenodoxin precursor to the mature form (Sagara *et al.*, 1984; Kumamoto *et al.*, 1985). The processing activity was inhibited by metal chelators such as EDTA, and reactivated by divalent ions such as Mn^{2+}. We carried out the purification of the processing protease according to the procedures as described in Materials and methods, and a typical result is summarized in Table I. Since the processing activity was not inhibited by serine protease inhibitors and microbial protease inhibitors (Sagara *et al.*, 1984), we added the inhibitors to the buffers used in preparing the matrix fraction to prevent the processing protease from degradation by lysozymal

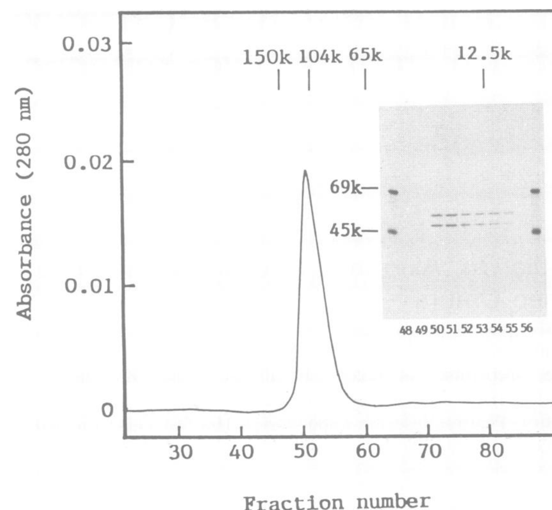

Fig. 2. FPLC Superose 12 gel filtration of the purified processing protease. Purified processing protease (30 μg) was applied to a FPLC Superose 12 column. The gel filtration was performed at a flow rate of 0.5 ml/min at 4°C with 20 mM NaPi buffer, pH 7.5, containing 50 mM NaCl. Protein was monitored at 280 nm, and 0.25 ml fractions were collected. The first 30 fractions that had no 280 nm absorbance are omitted in the figure. Molecular weight markers used in the gel filtration were: rabbit IgG (150 kd), yeast hexokinase (104 kd), human hemoglobin (65 kd), horse heart cytochrome *c* (12.5 kd). Fractions 48–56, which cover the 280 nm absorbance peak, were analyzed on an 8% polyacrylamide gel, silver-stained, and the results are shown in the insert. The two side lanes of the gel show the mol. wt markers: BSA (69 kd) and ovalbumin (45 kd).

proteases. The protease was purified >2300-fold from the matrix fraction, indicating a low content of the enzyme in mitochondria.

The final purified processing protease contained two polypeptides of 55 kd (P-55) and 52 kd (P-52) on SDS–PAGE (Figure 1, lane 7). The quantities of the two polypeptides were almost equal as revealed by densitometry. Another faint band was observed at 45 kd in some purified preparations as shown in Figure 1, lane 7. However, the third band could be removed from the processing protease by repeating FPLC Mono-Q chromatography without affecting the processing activity of the protease. P-55 and P-52 were not separated from each other at all chromatographic steps in the purification. Additionally, they were not dissociated by hydrophobic chromatography with aniline–Sepharose 4B, metal-chelating chromatography and FPLC Superose 12 chromatography. Because antibody to P-55 did not react with P-52, the smaller polypeptide was not the product degraded from the larger one.

The processing protease was estimated to be 105 kd as judged by Sephadex G-150 gel filtration. The behaviour of the protease on the gel filtration did not change even when the concentration of NaCl in the elution buffer was increased to 300 mM. When the purified processing protease was applied to gel filtration, the two polypeptides were recovered from the same fractions around the peak of processing activity showing a molecular mass of 105 kd (Figure 2). The results suggested that the processing protease is a dimer structure. As reported by Neupert and his co-workers (Hawlitschek *et al.*, 1988). the processing protease purified from *N.crassa* also consisted of two protein components of 57 kd (MPP) and 52 kd (PEP). However, MPP and PEP did not form a complex.

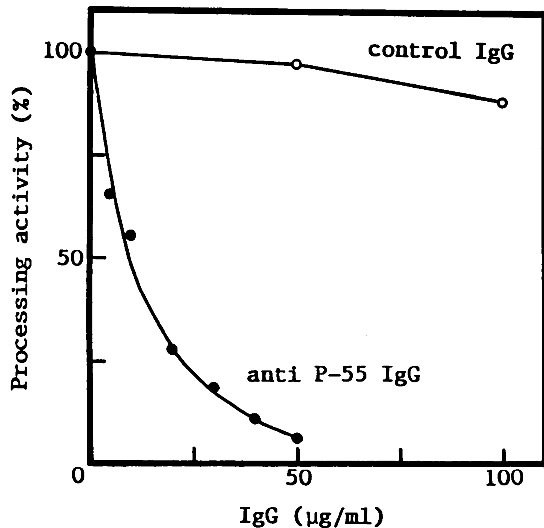

Fig. 3. Removal of processing activity from rat liver mitochondrial matrix by immunoadsorption with P-55 antibody. Rat liver mitochondrial matrix at a protein concentration of 1 mg/ml was incubated with increasing amounts of anti-P55 IgG or preimmune IgG at 4°C for 60 min. After protein A–Sepharose was added to each reaction mixture, the incubation was continued for 60 min at 4°C with gentle mixing. The reaction mixtures were centrifuged to precipitate the protein A–Sepharose. The resulted supernatants were assayed for processing activity to adrenodoxin precursor as described in Materials and methods. Processing activities are expressed in the figure as the percentages of the control without IgG addition.

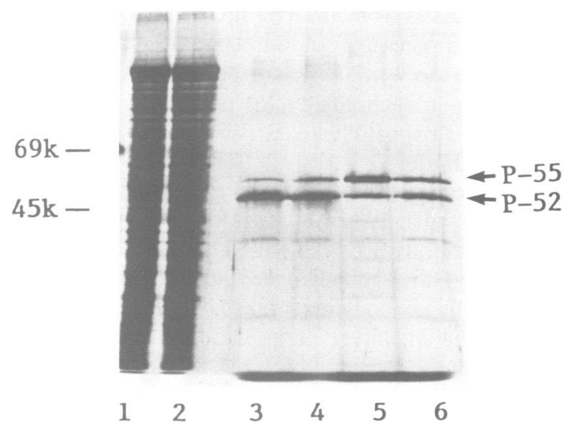

Fig. 4. Affinity chromatography of rat liver mitochondrial matrix with anti-P55 IgG affinity column. The preparation of anti-P55 IgG affinity column, and affinity chromatography of rat liver mitochondrial matrix were carried out as described in Materials and methods. Each eluted fraction was recovered, dialyzed completely against water and then lyophilized. The lyophilized samples were solubilized in SDS sample buffer, and subjected to SDS–PAGE using an 8% polyacrylamide gel followed by silver staining. **Lane 1**, matrix fraction; **lane 2**, fraction passed through the affinity column; **lane 3**, low salt eluate; **lane 4**, high salt eluate; **lane 5**, low pH eluate; **lane 6**, purified enzyme (FPLC Mono-Q II Fraction). BSA (69 kd) and ovalbumin (45 kd) were used as the mol. wt markers. P-55 and P-52 denotes the two subunits of the processing protease.

P-55 is an essential component of processing protease

The two polypeptides of the processing protease were efficiently separated by reverse-phase chromatography. However, neither of them showed processing activity, and their combination also failed to restore the activity (data not shown). Inactivation of the processing activity was possibly caused by the treatment with organic solvent. Antibody against P-55 recognized a single protein of 55 kd in an immunoblot of rat liver mitochondrial matrix, which was identical with the purified P-55. After incubation of rat liver mitochondrial matrix with the antibody, the processing activity was not significantly reduced. However, when the incubation of the matrix with the antibody was followed by the immunoprecipitation with protein A–Sepharose, the processing activity was lost from the supernatant, and the decrease of the processing activity was dependent on the amounts of the antibody added (Figure 3). On the other hand, the treatment of the matrix fraction with the control IgG and protein A–Sepharose did not precipitate the processing activity. These results indicated that P-55 is an essential component of the processing protease.

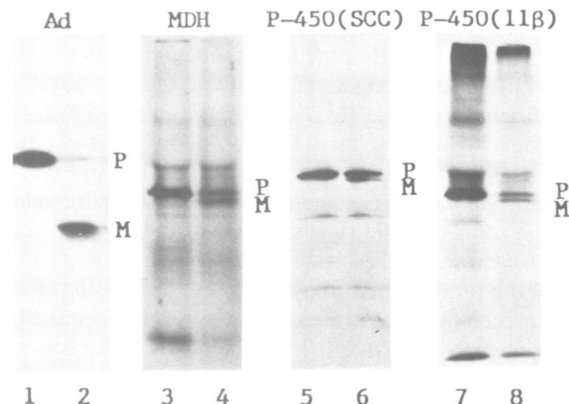

Fig. 5. Processing of various precursors by purified processing protease. Translation products containing [^{35}S]methionine-labeled precursors of adrenodoxin (Ad) (**lanes 1** and **2**), malate dehydrogenase (MDH) (**lanes 3** and **4**), P-450(SCC) (**lanes 5** and **6**) and P-450(11β) (**lanes 7** and **8**) were incubated in the absence of (**lanes 1**, **3**, **5** and **7**), or with (**lanes 2**, **4**, **6** and **8**) purified processing protease at 30°C for 30 min. After the reaction was stopped by addition of 5 mM EDTA, immunoprecipitation, SDS–PAGE and fluorography were carried out as described in Materials and methods. P and M in the figure denote precursors and mature forms respectively.

P-55 and P-52 form a complex

To determine whether or not P-55 and P-52 form a heterodimer complex, we prepared an anti-P55 IgG affinity column as described in Materials and methods. Matrix fraction from rat liver mitochondria was applied to the column. The column was washed first with a low salt buffer (150 mM NaCl), then with a high salt buffer (500 mM NaCl), and finally eluted with a low pH buffer (pH 2.5). The fraction passed through the column had little P-55 as determined by immunoblotting, and very low processing activity (data not shown), whereas no obvious changes were observed in protein composition (Figure 4, compare lane 1 with lane 2). When the column was eluted with the buffers of different salt concentrations, we found that the low salt eluate contained two polypeptides corresponding to P-55 and P-52 (Figure 4, lane 3). The amount of P-52 was ~10 times more than P-55. In the high salt eluate, the two polypeptides were also observed, but their contents were close (Figure 4, lane 4). P-55 was mainly eluted from the column with the low pH buffer (Figure 4, lane 5). Under the same conditions, we did not observe any binding of P-55 and P-52 to the control IgG column (data not shown). Although both low

and high salt eluates from anti-P55 IgG affinity column were active in the processing of adrenodoxin precursor, the activities were too weak to be correlated with the amounts of the proteins in each eluate. No processing activity was observed in the low pH eluate, possibly due to the treatment with low pH buffer.

Complete separation of P-55 and P-52 was not achieved by the immunoaffinity chromatography. Since we used a polyclonal antibody to P-55, P-55 was heterogeneously dissociated from the antibody under various ionic conditions. However, as the antibody recognized only P-55, the binding of P-52 to the affinity column indicated that the latter interacted with the former which was bound to the column. At increasing ionic concentration, P-52 was dissociated from P-55 in IgG affinity chromatography, but high ionic strength did not cause the dissociation of the two subunits in gel filtration. Our explanation is that high ionic strength weakens the interaction of the two subunits when they are bound to the antibody. These observations, combined with the result of the gel-filtration experiments, indicated that P-55 and P-52 form a heterodimer complex responsible for the processing activity.

Enzymatic properties of the purified processing protease

The purified protease cleaves several protein precursors. Various mitochondrial protein precursors are cleaved by matrix processing protease. We investigated the processing activity of the purified processing protease using several mitochondrial protein precursors, including the matrix-located proteins adrenodoxin (Nabi and Omura, 1980) and malate dehydrogenase (MDH) (Mihara *et al.*, 1982), and membrane-bound proteins P-450(SCC) (Dubois *et al.*, 1981) and P-450(11β) (Nabi *et al.*, 1980). As shown in Figure 5, the purified protease cleaved the precursors of adrenodoxin (lanes 1 and 2), MDH (lanes 3 and 4), P-450(SCC) (lanes 5 and 6) and P-450(11β) (lanes 7 and 8). Judging from the size of the proteins before and after the processing, all the tested precursors were processed to the mature forms. MDH has been reported to have an intermediate form that contained nine more amino acid residues than the mature form (Sztul *et al.*, 1988). However, we could not clearly distinguish the sizes between the intermediate and the mature form in SDS–PAGE. Although the processing was observed for all precursors tested, the processing efficiencies were quite different among the precursors. We compared the enzyme-dependent processing of adrenodoxin and P-450(SSC) precursors. The purified protease efficiently processed adrenodoxin precursor, but the processing of P-450(SCC) precursor was slow (Figure 6). In the presence of a sufficient amount of the protease, adrenodoxin precursor was completely processed, whereas only ~50% of P-450(SCC) precursor was converted to the mature form.

The purified protease is a metalloprotease. The purified processing protease was insensitive to PMSF and microbial protease inhibitors, including leupeptin and pepstatin, but sensitive to metal chelators such as EDTA and *o*-phenanthroline. The presence of Mn^{2+} stimulated the processing activity. The addition of 0.5 mM EDTA completely inhibited the enzyme activity (Figure 7, lane 2). *o*-Phenanthroline also inhibited the activity at 0.5 mM (data not shown). When the EDTA-inhibited protease was subsequently treated with excess $MnCl_2$, the enzyme was reactivated to normal levels (Figure 7, lanes 4 and 5). The results indicated that the processing protease is a metalloprotease. The enzyme activity was also sensitive to neutral salts including NaCl and KCl. In the presence of 150 mM NaCl or KCl the processing activity was almost completely inhibited (data not shown).

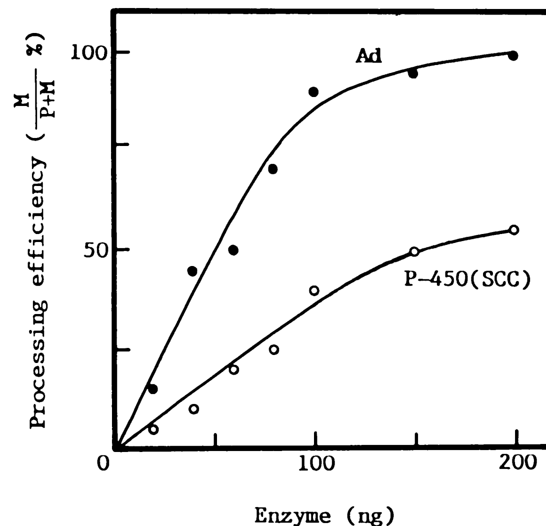

Fig. 6. Processing efficiencies of precursors of adrenodoxin and P-450(SCC). Processing of adrenodoxin (Ad) and P-450(SCC) precursors was carried out with increasing amounts of purified processing protease as indicated in the figure. Processing activities were assayed as described in Materials and methods, and are shown in the figure as the percentages of the conversion from the precursors to the mature forms.

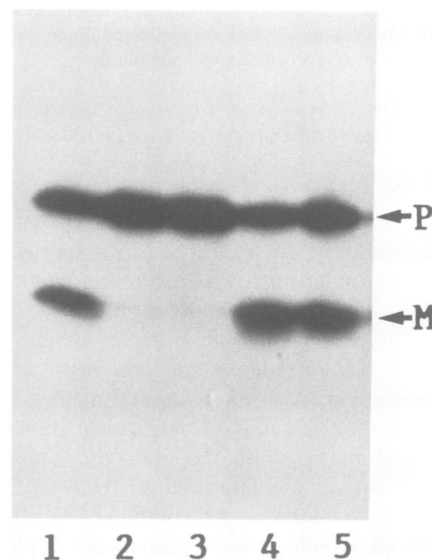

Fig. 7. Inhibition of processing activity by EDTA and reactivation by $MnCl_2$. Purified processing protease was preincubated with 0.5 mM (**lanes 2** and **4**), or 1 mM (**lanes 3** and **5**) ETDA, or in the absence of EDTA (**lane 1**) for 5 min. Subsequently, $MnCl_2$ was added at a concentration of 2 mM (**lanes 1**, **3** and **5**). After the treatment, the enzyme activity was assayed using adrenodoxin precursor as described in Materials and methods. P and M denote the precursor and the mature form respectively.

The purified protease is an endoprotease. Although the mitochondrial processing protease has often been described

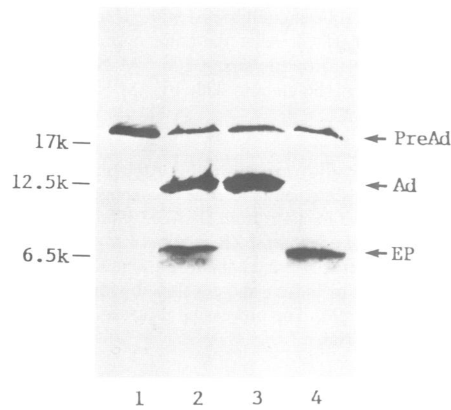

Fig. 8. Detection of the cleaved extension peptide of adrenodoxin precursor after the processing by purified processing protease. [^{3}H]leucine-labeled adrenodoxin precursor (**lane 1**) was incubated with the purified processing protease at 30°C for 30 min. The reaction mixture was divided into three aliquots. One was dissolved in SDS sample buffer (**lane 2**). The other two aliquots were immunoprecipitated with adrenodoxin antibody (**lane 3**), and antibody to the extension peptide (**lane 4**). The immunoprecipitates were dissolved in SDS sample buffer, and all the samples were analyzed by SDS−PAGE with 18% polyacrylamide gel followed by fluorography as described in Materials and methods. PreAd denotes adrenodoxin precursor; Ad, mature adrenodoxin; EP, adrenodoxin extension peptide. The mol. wt markers are: myosin (17 kd), cytochrome *c* (12.5 kd) and aprotinin (6.5 kd).

as an endoprotease in previous studies (McAda and Douglas, 1982), only one of the processed products—the mature form—has been detected so far. Therefore, the question of whether the extension peptide is cleaved in small fragments or *en bloc* has remained unanswered. Since mitochondria contain various other proteases, the extension peptides could be too short lived to be detected in mitochondria, even if it was cleaved *en bloc* by the processing protease. The availability of the purified processing protease enabled us to detect the intact extension peptide after it was cleaved from the precursor.

Fortunately, the adrenodoxin precursor has a relatively large extension peptide of 6 kd, consisting of 58 amino acid residues (Okamura *et al.*, 1985). Because there is only one methionine but eight leucines in the extension peptide, we used [^{3}H]leucine as the label in synthesizing the precursor. The [^{3}H]leucine-labeled precursor was processed with the purified protease, and analyzed on SDS−PAGE followed by fluorography. In addition to adrenodoxin precursor and the mature form, another small peptide of ~7 kd was observed below the mature form (Figure 8, lane 2), which was a little larger than the size of the intact extension peptide of adrenodoxin precursor. Because the extension peptide was rich in positive charges, the slow migration on the gel had been expected. To identify the 7 kd peptide with the extension peptide, we prepared an antibody against the amino- and carboxy-terminal portions of the extension peptide (anti-EP). The specificity of anti-EP to the extension peptide of adrenodoxin precursor was confirmed by immunoprecipitation of *in vitro* synthesized adrenodoxin precursor and the mature form. Anti-EP reacted with the precursor but not with the mature form. When adrenodoxin precursor was processed *in vitro* with the purified processing protease and immunoprecipitated with anti-EP, we found that the antibody precipitated both the precursor and the 7 kd peptide, whereas mature adrenodoxin did not react with the antibody (Figure 8, lane 4). On the other hand, the antibody against mature adrenodoxin did not precipitate the 7 kd peptide (Figure 8, lane 3). The reactivities with the antibodies and the size on the SDS−PAGE indicated that the detected 7 kd peptide was indeed the intact extension peptide of adrenodoxin. The detection of the intact extension peptide after the processing suggested that the processing by the processing protease is an endo-type cleavage.

Discussion

In eukaryotic cells, mitochondria contain a machinery for protein import, in which multiple factors seem to be involved. Intensive studies have been carried out on the mechanism of the import process and processing protease is the first clearly identified protein involved in the process. Processing protease from *Neurospora* contained two protein components, MPP and PEP. Although both of them were required for the processing activity, they did not form a complex. We have shown that the processing protease purified from rat liver mitochondria was also composed of two subunits, P-55 and P-52, which were very close in mol. wt to MPP and PEP, respectively. Unlike the fungi enzyme, however, P-55 and P-52 were tightly associated to form a heterodimer complex. The interaction between the two subunits was weakened by increasing ionic strength. It has been observed that the two products of yeast *MAS1* and *MAS2* genes, which were related to the two subunits of the matrix processing protease, dissociated in gel filtration, but remained associated in sucrose gradient centrifugation (Yang *et al.*, 1988). It seems that the interaction of the two subunits is different between rat enzyme and lower eukaryotes' enzymes. It is too early to draw a conclusion that the two subunits of the rat processing protease are equivalent to MPP and PEP of *Neurospora* enzyme. Comparison of the antigenicities and primary amino acid sequences of the two enzymes is needed.

We present here the first evidence that the mitochondrial processing protease is an endoprotease. After the processing of adrenodoxin precursor by the purified protease, both of the processing products—mature form and cleaved extension peptide—were detected on SDS−PAGE, suggesting that the processing of the precursor was an endo-type cleavage. Endo-type cleavages have also been observed for the precursors of secretory proteins of eukaryotes (Mollay *et al.*, 1982), and exported proteins of *Escherichia coli* (Suzuki *et al.*, 1988). It appears that the endoproteolysis of precursors is common for all the signal cleaving proteases. In mitochondrial matrix, some precursors are cleaved by two steps. Kalousek *et al.* (1988) identified two processing enzymes in rat liver mitochondria, which sequentially cleaved the precursors of ornithine carbamoyltransferase (OCT) and MDH. We also examined the processing of OCT precursor by the purified processing protease using an antibody to rat OCT (kindly provided by Dr M.Mori of Kumamoto University) and found that the protease cleaved the precursor to the intermediate form (data not shown). It appears that our enzyme corresponds to protease I described by Kalousek *et al.*, but our enzyme was strongly inhibited by metal chelators whereas protease I was not.

Since complete separation of the two subunits, P-55 and P-52, in active forms has not been successful, the functions of each subunit have remained unclear. As MPP alone had

low processing activity that was enhanced by PEP, it was proposed that MPP was a real proteolytic subunit, and PEP was involved in the binding to the extension peptides of the precursors so that MPP could efficiently and correctly cleave the precursors. It is very interesting that *Neurospora* cells contained a 15-fold excess of PEP over MPP, and 75% of PEP was found to be associated with mitochondrial inner membrane. These observations implicated the regulatory function of PEP. A general structural feature of the extension peptides seems to be the formation of amphiphilic helix, which favours the binding of the precursors to mitochondria and their translocation across the mitochondrial membranes (Ito *et al.*, 1985; Roise *et al.*, 1986; von Heijne; 1986), but no information is yet available concerning the recognition of the extension peptides by the processing protease.

At isotonic concentrations of NaCl or KCl, the purified processing protease as well as the matrix fraction was almost inactive. However, mitochondria can carry out the import and processing of various protein precursors under the same conditions. The real concentrations of various ions in mitochondria at different respiratory states could be variable, and the salt-dependent inactivation of the processing protease may be one of the regulatory mechanisms of the enzyme. Since *Neurospora* enzyme that was not a complex of the subunits was similarly sensitive to the salts (Hawlitschek *et al.*, 1988), we prefer an interpretation that salt concentrations affect the interaction between the enzyme and the substrates rather than that between the two subunits.

Recent studies have implicated some metalloproteases in a number of important intracellular processes. Membrane-bound metalloproteases have been found in animal cells, which play important roles in intracellular vesicular transport including endocytosis (Jochen and Berhanu, 1986; Strous *et al.*, 1988) and exocytosis (Mundy and Strittmatter, 1985) as well as the transport between endoplasmic reticulum and Golgi complex (Strous *et al.*, 1988). Mitochondrial processing protease is a soluble metalloprotease, and participates in the transport of protein precursors into the organelles. Further studies on the catalytic mechanism and primary structure of the mitochondrial processing protease will contribute to the elucidation of the functions of metalloproteases in various intracellular events in eukaryotic cells.

Materials and methods

Preparation of mitochondrial matrix fraction

All manipulations of purification were carried out at 4°C. Fresh livers (4 kg) from Wister rats were homogenized in the isolation buffer containing 10 mM NaPi, pH 7.5, 0.25 M sucrose and 200 mM KCl with a Waring blender. The homogenate was centrifuged at 500 *g* for 10 min. The precipitate was homogenized again in the isolation buffer with a glass–Teflon homogenizer and centrifuged as above. The supernatants were combined and centrifuged at 9000 *g* for 10 min to obtain the pellet of mitochondria. The mitochondria were washed once and then suspended in a hypotonic buffer containing 20 mM NaPi, pH 7.0, 2 μg/ml each of leupeptin and pepstatin and 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and gently stirred for 30 min to lyse the contaminating lysosomes. The mitochondria were recovered by centrifugation at 10 000 *g* for 15 min, resuspended in the hypotonic buffer and sonicated with a Branson sonicator. The sonicated mitochondrial suspension was adjusted to 0.1 M NaCl by adding solid NaCl, and centrifuged at 105 000 *g* for 60 min. The supernatant was used as the matrix fraction.

Purification of processing protease

DEAE–cellulose chromatography. The matrix fraction was diluted 2-fold with cold water, and loaded to a DEAE cellulose column (Whatman DE-52; 7.0 × 40 cm) which had been equilibrated with 10 mM NaPi buffer, pH 7.0, containing 50 mM NaCl. After the column was fully washed with 10 mM NaPi buffer, pH 7.0, containing 100 mM NaCl, the processing protease was eluted from the column with 10 mM NaPi buffer, pH 7.0, containing 150 mM NaCl.

Aminohexyl–Sepharose 4B chromatography. Aminohexyl–Sepharose 4B was prepared by conjugating diaminohexane to cyanogen bromide-activated Sepharose 4B. The active fractions from DEAE–cellulose were pooled and applied to an aminohexyl–Sepharose 4B column (3.2 × 35 cm) equilibrated with 10 mM NaPi buffer, pH 7.0, containing 200 mM NaCl. The column was washed with the same buffer, and developed with a linear gradient of NaCl from 200 to 600 mM. The processing protease was eluted from the column at ~400 mM NaCl.

Sephadex G-150 gel filtration. The fractions containing processing activity were pooled, concentrated to ~20 ml by ultrafilters (Immersible CX-30, Millipore Co.), and then loaded to a Sephadex G-150 column (Pharmacia Chemical Co.; 4.0 × 95 cm). The gel filtration was performed in 20 mM NaPi buffer, pH 7.0, at a flow rate of 25 ml/h.

Hydroxylapatite chromatography. The active fractions from Sephadex G-150 were collected, and applied to a hydroxylapatite column (Bio-Rad Lab.; 1.2 × 15 cm) equilibrated with 20 mM NaPi buffer, pH 7.0. The column was washed with 50 mM NaPi buffer, pH 7.0, and elution was carried out in a linear gradient of NaPi buffer, pH 7.0, from 50 to 200 mM. The processing protease was eluted at ~100 mM NaPi.

FPLC Mono-Q chromatography. The active fractions from hydroxylapatite were loaded to a Mono-Q anion-exchange column (Pharmacia Chemical Co.; HR5/5), which had been equilibrated with 10 mM NaPi buffer, pH 7.0, containing 50 mM NaCl at a flow rate of 1 ml/min. After washing the column with 10 mM NaPi buffer, pH 7.0, containing 100 mM NaCl, elution was carried out in a linear gradient of NaCl from 100 to 250 mM. The processing protease was eluted from the column at 170 mM NaCl. The Mono-Q chromatography was performed once more and the eluted active fractions contained the pure processing protease, which consisted of two polypeptides, P-55 and P-52.

Preparation of mitochondrial protein precursors

Mitochondrial protein precursors were synthesized in rabbit reticulocyte lysates using [^{35}S]methionine as the label (Pelham and Jackson, 1976). mRNA of adrenodoxin and P-450(SCC) were prepared by *in vitro* transcription from the cDNAs cloned into SP-6 transcription vectors (Ou *et al.*, 1986; Furuya *et al.*, 1987). Precursors of P-450(11β) and MDH were synthesized *in vitro* by using total RNA prepared from bovine adrenal cortex and rat liver respectively (Ou *et al.*, 1988). *In vitro* transcription and translation were carried out as described previously (Ou *et al.*, 1986).

Assay of processing protease activity

Translation products containing mitochondrial protein precursors were incubated with processing protease fractions in 20 mM Hepes buffer, pH 7.6, containing 0.5 mM $MnCl_2$, 2 μg/ml each of leupeptin and pepstatin, 0.1 mM PMSF and 0.1% Tween 20 at 30°C for 20 min. The processing reaction was stopped by addition of EDTA to a final concentration of 5 mM. The precursors and mature forms were immunoprecipitated with specific antibodies and analyzed by SDS–PAGE followed by fluorography. The optical densities of the bands on the fluorogram corresponding to precursors and mature forms were determined by a densitometer (Shimadzu Chromatoscanner CS-930). The processing activities were quantitated by the conversion of the precursors to mature forms. Adrenodoxin precursor was usually used as the substrate in determining the activity of the processing protease. One unit of the enzyme activity was defined as the amount that produced 50% conversion of adrenodoxin precursor to the mature form under the above assay condition.

Preparation of specific antibodies

Antibody to P-55 (anti-P55). Purified processing protease, which consisted of two protein components, was applied to a reverse-phase C_4 column. The column was eluted with a linear gradient of acetonitrile from 20 to 80% in 0.1% trifluoroacetic acid (TFA). The processing protease was separated into two protein components, P-55 and P-52, which were separately collected and concentrated. P-55 (50 μg) was mixed with Ribi adjuvant (Ribi Immunochem. Res. Inc.) and half of the mixture was injected s.c. into a rabbit. The injection of the other half into the same animal was performed 4 weeks later. One week after the second injection the rabbit was bled.

Immunoglobulin was prepared from collected serum by ammonium sulfate precipitation followed by DEAE−cellulose chromatography.

Antibody to the extension peptide of adrenodoxin precursor (anti-EP). Two 15 mer peptides, MAARLLRVASAALGC and AVATRTLSVSGRANC, were synthesized on an automated peptide synthesizer (Applied Biosystems Model 430A) using t-Boc solid-phase method. The structures of the two peptides correspond to the sequences of the amino- and carboxy-terminal portions respectively, of the extension peptide of adrenodoxin precursor with an additional carboxy-terminal cysteinyl residue. After the syntheses, the peptides were cleaved from the resin, deprotected by anhydrous hydrogen fluoride and purified to >90% purity by a C_{18} reverse-phase column. The sequences were confirmed by Edman degradation using an automated peptide sequencer (Applied Biosys. Model 477A). To enhance the production of antibodies to the peptides, the peptides were coupled to BSA with *N*-(ϵ-maleimidocaproxyloxy)succinimide (EMCS) utilizing the cysteinyl residues in the peptides as follows. BSA (6 mg) was suspended in 1 ml of 0.1 M NaPi buffer, pH 7.0. EMCS (DOJIN) was dissolved in dimethylformamide and added to the BSA solution at a final concentration of 4 mM. After incubation at 30°C for 30 min, the mixture was passed through a Sephadex G-25 column to remove excess EMCS. Two milligrams each of the peptides was dissolved in a small volume of 0.1 M NaPi buffer, pH 7.0, and added to the activated BSA solution. The coupling reaction was carried out at 30°C for 5 h. Mercaptoethanol (100 μl) was added to the reaction mixtures to stop the coupling reaction. About 20 molecules of the peptides were coupled to each molecule of BSA and the coupling efficiency was >95% as checked by SDS−PAGE. Peptide−BSA complex (1 mg) was mixed with Freund's complete adjuvant (DIFCO) and injected s.c. to a rabbit. After 1 month, a second injection was made with 0.5 mg antigen mixed with the complete adjuvant. The rabbit was bled 1 week later.

Anti-P55 IgG affinity chromatography

Total IgG was prepared from anti-P55 serum as described above. Specific anti-P55 IgG was purified by adsorption to partially purified processing protease. The purified IgG was coupled to Affigel 10 (Bio-Rad Lab.) at 1 ml of the IgG (5 mg/ml) to 1 ml swollen resin. A control IgG affinity column was prepared by coupling the immunoglobulin from a preimmune rabbit to the resin under the same condition. All the procedures for affinity chromatography were carried out at room temperature. The IgG affinity column (5 ml) was prewashed with 2 vol of 20 mM Hepes buffer, pH 7.5, containing 1 M NaCl, and then with 2 vol of 0.1 M glycine−HCl buffer, pH 2.5. The column was subsequently equilibrated with a equilibration buffer containing 20 mM Hepes, pH 7.5, and 0.1% Tween 20. Rat liver mitochondrial matrix fraction (50 mg) was applied to the column. After washing the column with 10 vol of the equilibration buffer containing 50 mM NaCl, elution was carried out first with 2 vol of the equilibration buffer containing 0.15 M NaCl, and then with 2 vol of the equilibration buffer containing 0.5 M NaCl. Complete elution of adsorbed proteins from the column was performed with 0.1 M glycine−HCl buffer, pH 2.5. Eluted fractions were desalted and concentrated. The proteins in the fractions were analyzed by SDS−PAGE followed by silver staining.

Detection of cleaved extension peptide of adrenodoxin precursor after the processing

Adrenodoxin precursor was synthesized as described above except that [^{3}H]leucine was used as the label and purified by immunoadsorption to adrenodoxin antibody. The purified adrenodoxin precursor was incubated with purified processing protease (300 ng) in a buffer containing 10 mM Hepes, pH 7.5, 0.5 mM $MnCl_2$, 0.1% Tween 20, 2 μg/ml each of leupeptin and pepstatin and 0.5 mM PMSF at 30°C for 30 min. After EDTA was added to the mixture to stop the reaction. The reaction mixture was divided into three aliquots. One portion was dissolved in SDS sample buffer. The other two aliquots were subjected to immunoprecipitation with adrenodoxin antibody and anti-EP antibody respectively. The three samples were analyzed on 18% polyacrylamide gel. After the electrophoresis, the proteins were transferred to a Zeta membrane (Bio-Rad Lab.), which can tightly bind small polypeptides. The membrane was dried at 80°C for 15 min, and then soaked in 15% PPO−toluene solution for 5 min. The PPO-treated membrane was dried, and exposed to a X-ray film at −80°C.

Analytical methods

Protein was determined according to the method of Lowry by using BSA as the standard (Lowry *et al.*, 1951). Immunoprecipitation was carried out as described by Ono and Ito (1984a) except that protein A−Sepharose was used as the immunoadsorbent. SDS−PAGE was carried out as described by Laemmli (1970). Fluorography was carried out by the DMSO−PPO method (Raskey and Mills, 1975).

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