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Mitochondrial Protein Import: Identification of Processing Peptidase and of PEP, a Processing Enhancing Protein

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Summary

Transport of nuclear-encoded precursor proteins into mitochondria includes proteolytic cleavage of amino-terminal targeting sequences in the mitochondrial matrix. We have isolated the processing activity from Neurospora crassa. The final preparation (enriched ca. 10,000-fold over cell extracts) consists of two proteins, the matrix processing peptidase (MPP, 57 kd) and a processing enhancing protein (PEP, 52 kd). The two components were isolated as monomers. PEP is about 15-fold more abundant in mitochondria than MPP. It is partly associated with the inner membrane, while MPP is soluble in the matrix. MPP alone has a low processing activity whereas PEP alone has no apparent activity. Upon recombining both, full processing activity is restored. Our data indicate that MPP contains the catalytic site and that PEP has an enhancing function. The mitochondrial processing enzyme appears to represent a new type of "signal peptidase," different from the bacterial leader peptidase and the signal peptidase of the endoplasmic reticulum.

Introduction

Most nuclear-encoded precursors of mitochondrial proteins contain amino-terminal presequences (for reviews see Pfanner and Neupert, 1987; Nicholson and Neupert, 1988). These presequences are required for the precursors to enter the mitochondrial matrix, where they are proteolytically removed (Hurt et al., 1984; Horwich et al., 1985, 1986; Emr et al., 1986; Keng et al., 1986; Grivell et al., 1987; Vassarotti et al., 1987a). This cleavage is not essential for completing import but is necessary for further assembly of the newly imported polypeptides into functional complexes (Zwizinski and Neupert, 1983; Lewin and Norman, 1983; Ou et al., 1986; Hartl et al., 1986, 1987). Precursor targeting sequences differ considerably in their structures. One of the few common themes is the high content of positively charged amino acids and of hydroxylated amino acids. Presequences may form amphipathic structure in the form of either α-helices or β-sheets (von Heijne, 1986; Roise et al., 1986; Vassarotti et al., 1987b). Despite the large variability of the sequences of mitochondrial leader peptides, relatively minor alterations of the presequence can prevent cleavage by the processing peptidase (Hurt et al., 1987; Grivell, 1987; Nguyen et al., 1987). This suggests that distinct, but up to now undefined, structural elements are required for cleavage. Similarly, the cleavage sites show wide variation among different precursors of a single organism and among precursors of different organisms (for review see Nicholson and Neupert, 1988).

The enzyme responsible for the cleavage of presequences was initially found in the matrix of yeast mitochondria (Böhni et al., 1980; McAda and Douglas, 1982) and later also in the mitochondrial matrix of other organisms (Miura et al., 1982; Conboy et al., 1982; Schmidt et al., 1984). This matrix protease, or processing peptidase, is a soluble protein in both mitochondria and the chloroplast stroma (Robinson and Ellis, 1984). It is different from the signal peptidase or leader peptidase that cleaves off the amino-terminal signal sequences from proteins being translocated across the membranes of the endoplasmic reticulum (Evans et al., 1986) or across the plasma membrane of bacteria (Zwizinski and Wickner, 1980; Dev and Ray, 1984), respectively. The processing peptidase from mitochondria (and from chloroplasts) is dependent on divalent metal ions such as Mn^{2+}, Zn^{2+}, or Co^{2+} and is inhibited by metal chelators such as EDTA and orthophenanthroline or bathophenanthroline. It is therefore assumed that the enzyme is a metalloprotease.

The processing peptidase has been partially purified from different sources (McAda and Douglas, 1982; Böhni et al., 1983; Kumamoto et al., 1986). Molecular characterization of the pure enzyme, however, is required to answer a number of questions concerning mitochondrial biogenesis: Is there only a single enzyme for the hundreds of different precursor proteins? If so, how can it recognize the extreme variety of presequences and cleavage sites? Does the enzyme perform only a single peptide-bond cleavage during removal of the signal peptide? What is its evolutionary relationship to other peptidases? How is the peptidase itself synthesized and processed in the course of its import?

Here we describe the purification of the processing enzyme, starting with whole-cell extracts from Neurospora crassa. Two different proteins were found to be required for full enzyme activity, namely the processing peptidase (MPP, 57 kd) and a processing enhancing protein (PEP, 52 kd). They did not form a detectable complex. MPP and PEP were readily resolved with most chromatographic procedures applied. This appears to explain the low yields of processing activity during purification and the difficulty in purifying the enzyme. Upon mixing both polypeptides, activity was restored. The MPP component appears to bear the catalytic activity, which is stimulated by PEP. The two proteins are present in unequal molar amounts, PEP being about 15-fold more abundant. In contrast to the MPP polypeptide, which is completely soluble in the matrix, PEP is partly associated with the inner surface of the inner membrane. We suggest that the two proteins cooperate in binding precursors and proteolytic cleavage. The cDNA sequence of PEP is also reported.

Table 1. Purification of Mitochondrial Processing Peptidase

Fraction	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification	Yield (%)
Total extract	1,320	18,414	57,440	3.1	1	100
DEAE-cellulose	460	1,996	43,080	22	7.1	75
Zn-chelate	230	251	32,166	128	41	56
PEI-cellulose	150	–	16,083	–	–	28
PEG precipitation	10.0	5.13	6,893	1,344	434	12
Ecteola-cellulose	1.0	0.11	3,274	29,764	9,601	5.7

The processing peptidase of Neurospora mitochondria was isolated by subjecting a total cell extract to the purification steps indicated (see Experimental Procedures). One unit of activity was defined as that amount of enzyme catalyzing the cleavage of 1 fmol of precursor per min at 25°C. For each fraction obtained, total volume, protein content, and activity were measured, and specific activities, purification factors, and yields were calculated. Because of interference of PEI with the protein assay, we were unable to determine the protein content of the fraction obtained after PEI-cellulose chromatography.

Results

Purification of Processing Enzyme

Table 1 summarizes the purification of the processing activity from a Neurospora cell extract. The activity was tested with precursors of subunit 2 and subunit 9 of ATP synthase. We define 1 U of activity as that amount of enzyme which cleaves 1 fmol of precursor per min at 25°C. The cell extract was subjected sequentially to DEAE-cellulose chromatography, Zn-chelate chromatography, and polyethyleneimine (PEI)-cellulose chromatography. After precipitation of the activity with polyethylene glycol (PEG), a final chromatographic step was performed on Ecteola-cellulose. The purification was about 10,000-fold and the final yield was ca. 6%. The various purification steps were monitored by SDS–polyacrylamide gel electrophoresis and staining of protein bands with Coomassie blue (Figure 1). The final preparation consisted of two polypeptides, with apparent molecular weights of 57,000 and 52,000. As revealed by densitometry, the 52 kd polypeptide was in ca. 1.6-fold excess over the 57 kd component.

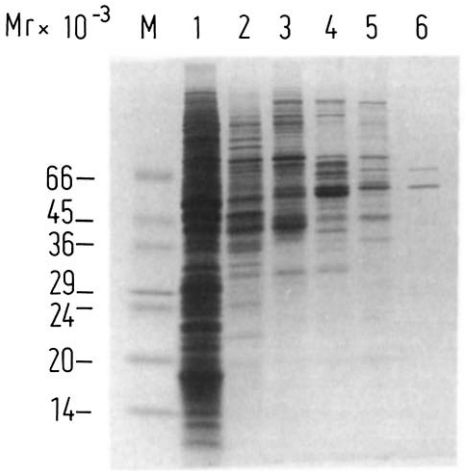

Figure 1. Purification of Mitochondrial Processing Enzyme: SDS–Polyacrylamide Gel Electrophoresis of Different Steps

Aliquots of different fractions of the processing enzyme purification were lyophilized. The residues were treated with 50% methanol, and precipitated protein was centrifuged, solubilized in SDS-containing buffer, and electrophoresed on a 16% polyacrylamide gel. Proteins were stained with Coomassie blue R-250. Lane 1, total cell extract (350 μg); lane 2, eluate of DEAE-cellulose chromatography (32 μg); lane 3, eluate of Zn-chelate chromatography (38 μg); lane 4, eluate of PEI-cellulose chromatography (30 μg); lane 5, pellet of PEG precipitation (25 μg); lane 6, eluate of Ecteola-cellulose chromatography (3 μg).

The 57 kd and 52 kd Proteins Are Associated with Processing Activity but Do Not Form a Complex

The enzyme preparation obtained after Zn-chelate chromatography was subjected to FPLC gel filtration on Superose 12. Later steps were not suitable for molecular sieving since the presence of detergent considerably changed the behavior of the 57 kd and 52 kd polypeptides on such a column. Gel electrophoresis and immunoblotting (see below) of the FPLC fractions revealed a partial separation of the 57 kd and 52 kd polypeptides. This demonstrates that they did not form a complex under the conditions of the gel filtration. Each protein migrated at its apparent monomeric molecular weight (Figure 2A). This was observed at low (50 mM NaCl) and at high (300 mM NaCl) salt concentrations. When the enzymatic activity of the various fractions was measured, the peak catalytic activity did not coincide with either protein peak (Figure 2B, panel a). Moreover, the yield of activity over the whole gradient was only 10%. This suggests that both the 57 kd and 52 kd polypeptides are necessary for full activity. To test whether two essential factors were being resolved, selected fractions obtained by FPLC gel filtration were mixed and enzyme activity was determined (Figure 2B). Fractions 8–10, which contained 52 kd polypeptide but little or no 57 kd polypeptide, were able to stimulate activity in fractions 4–6, which contained the 57 kd polypeptide (Figure 2B, panels b–d). Likewise, when fractions 4 or 5, containing mainly 57 kd polypeptide, were added to the various gradient fractions, high enzyme activities were found in 52 kd polypeptide–containing fractions 8–10 (not shown). We conclude from these data that both the 57 kd and the 52 kd polypeptides are required for enzyme activity, although they are apparently not present in a complex.

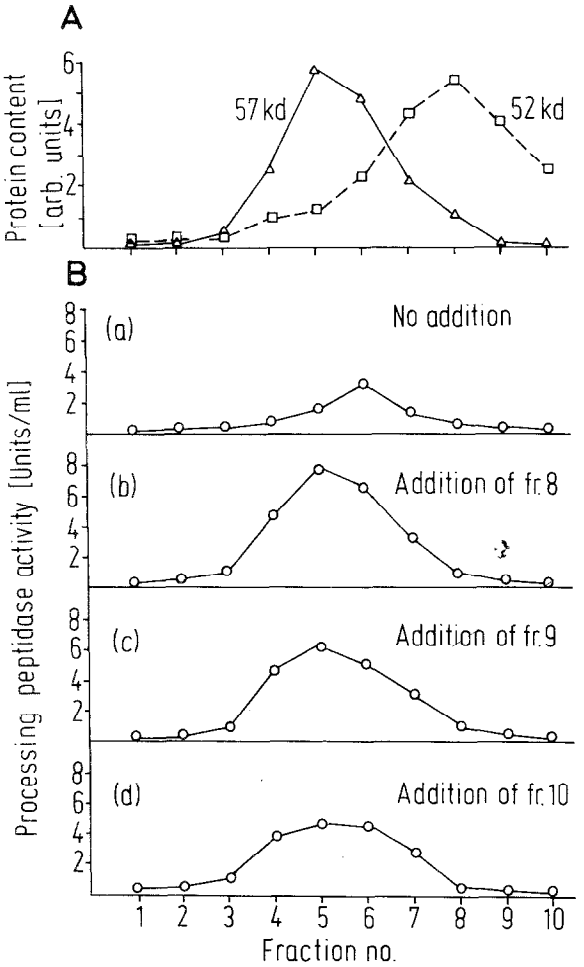

Figure 2. Gel Chromatography of the Eluate of Zn-Chelate Chromatography

Five hundred microliters of the eluate of Zn-chelate chromatography was chromatographed on a Superose 12 column as described in Experimental Procedures. Fractions of 250 μl were collected.

(A) Aliquots of each fraction were precipitated with trichloroacetic acid (TCA) and analyzed by SDS–polyacrylamide gel electrophoresis and Western blotting. The elution profiles of the 52 kd and 57 kd proteins were determined by immunostaining with antisera directed against both proteins using [^{14}C]protein A as a label and autoradiography. Quantification was carried out by laser densitometry.

(B) Ten microliters of each fraction was tested for processing peptidase activity as described in Experimental Procedures. (a) shows the elution profile of the processing activity without any addition of other fractions. In (b–d), 10 μl of fraction 8 (b), fraction 9 (c), or fraction 10 (d) was included in the assay.

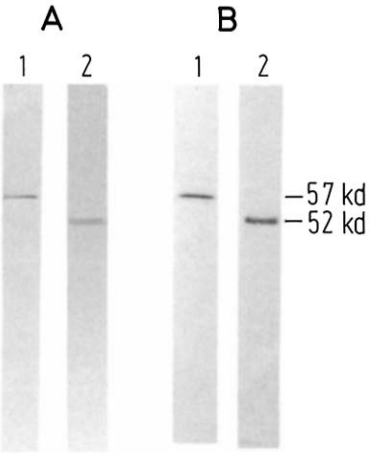

Figure 3. Immunoprecipitation and Immunoblotting of the 57 kd and 52 kd Polypeptides from a Mitochondrial Extract

(A) Mitochondrial pellets (100 μg) were solubilized in 30 mM Tris–HCl (pH 8.2), 300 mM NaCl, 1% Triton X-100. The extracts were electrophoresed on a 12.5% polyacrylamide–SDS gel and electrotransferred to nitrocellulose paper (Burnette, 1981). Immunolabeling was done with antisera against the 57 kd polypeptide (lane 1) and against the 52 kd polypeptide (lane 2). Bound antibodies were visualized by decoration with [^{14}C]protein A and autoradiography.

(B) Mitochondria were isolated from Neurospora cells grown in the presence of [^{35}S]sulfate, and mitochondrial extracts were prepared as in (A). The 57 kd component (lane 1) and 52 kd component (lane 2) were immunoprecipitated from extracts corresponding to 100 μg and 5 μg of mitochondria, respectively. Precipitates were dissociated in SDS-containing buffer and analyzed by electrophoresis and fluorography.

The 52 kd Component Is about 15-Fold More Abundant in Mitochondria Than the 57 kd Component

We prepared antibodies against the 57 kd and the 52 kd polypeptides by blotting the final preparation after gel electrophoresis to nitrocellulose and by immunizing rabbits with the individual bands cut from the nitrocellulose paper. The specificities of the antibodies obtained are demonstrated in Figures 3A and 3B. On a Western blot of a total extract from Neurospora mitochondria, the 57 kd antibody recognized only the 57 kd protein, and the 52 kd antibody recognized only the 52 kd protein (Figure 3A). Furthermore, direct immunoprecipitation from mitochondrial extracts led to selective precipitation of the 57 kd and 52 kd polypeptides (Figure 3B).

To determine quantitatively the amounts of 57 kd protein and 52 kd protein in mitochondria, we performed immunotitration employing mitochondria from Neurospora cells grown in the presence of [^{35}S]sulfate or [^{3}H]leucine. As judged from radioactivity in the immunoprecipitate, the 57 kd polypeptide is 0.03% of total mitochondrial protein, whereas the 52 kd protein is 0.4%–0.45%. Thus the 52 kd component is roughly 15 times more abundant than the 57 kd polypeptide. The relative content of 57 kd polypeptide in mitochondria of 0.03% corresponds to a relative content in whole cells of about 0.006%. This compares well with the purification factor of the final preparation of the processing enzyme (see Table 1). The relative cellular contents of the 57 kd and 52 kd polypeptides, as measured by immunoprecipitation, were found to agree with the relative frequencies of cDNA clones for the two polypeptides in a Neurospora cDNA library (Tropschug and Neupert, unpublished). Based on the content of 57 kd and 52 kd polypeptides in the total mitochondrial extract and their relative proportions in the purified preparation, the final yields of 57 kd and 52 kd polypeptides are 4% and 0.5%, respectively.

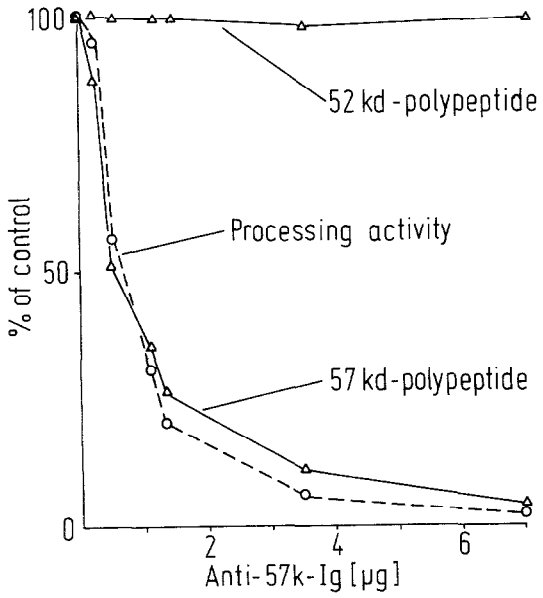

Figure 4. Removal of the 57 kd Component and of Processing Activity from a Mitochondrial Extract by Anti-57 kd Antibodies

Increasing amounts of immune globulins directed against the 57 kd polypeptide together with decreasing amounts of preimmune globulins were prebound to protein A–Sepharose. The samples thus contained roughly the same amounts of bound globulins, and the control contained only preimmune globulins. Mitochondrial pellets (20 µg) were lysed as in Figure 3, and the 57 kd component was immunoadsorbed to the Sepharose beads by end-over-end mixing at 4°C for 90 min. The beads were collected by centrifugation, and one-half of the supernatant was tested for processing activity. The other half was TCA-precipitated, electrophoresed on a 16% polyacrylamide gel under nonreducing conditions, and electrotransferred to nitrocellulose paper. Immunodetection of the 57 kd polypeptide was carried out as in Figure 3. Quantification was by laser densitometry. Activity (dashed line) and amounts of the proteins (solid lines) are expressed as percentages of the corresponding totals (control).

The 57 kd Polypeptide Is Limiting for Processing Activity in Mitochondrial Extracts

Antibodies were used to titrate the processing activity in mitochondrial extracts. Isolated mitochondria were lysed with Triton X-100. Increasing amounts of anti-57 kd or anti-52 kd antibodies prebound to protein A–Sepharose were added to aliquots of the extracts. After centrifugation, the supernatants were collected, and the 57 kd and 52 kd polypeptides and enzyme activity remaining in the supernatant were determined. As shown in Figure 4, the 57 kd polypeptide and the enzyme activity were removed from the lysed mitochondria with the anti-57 kd antibody in a parallel manner, while the 52 kd component stayed in the supernatant. Up to 50% of the 52 kd polypeptide was removed from the lysed mitochondria by the anti-52 kd antibody, but this was not accompanied by a decrease in the enzyme activity (not shown). The effect of complete depletion of 52 kd polypeptide from the mitochondrial extracts could not be measured in these experiments, since antibody amounts needed to achieve this led to unspecific inhibition of processing activity, an effect also observed with corresponding amounts of preimmune globulins. These data demonstrate that the 57 kd and the 52 kd polypeptides are separate components in crude mitochondrial extracts and that the 57 kd polypeptide is limiting for processing activity. The 52 kd polypeptide is not limiting for activity, probably because it is present in higher molar amounts.

Proteolytic Activity Is Associated with the 57 kd Component

When a Triton X-100 extract from mitochondria or a purified preparation of the processing enzyme (not shown) was incubated with antibody against the 57 kd component, the enzyme activity was not diminished. The same was true when preimmune serum was used (Figure 5A, columns 1 and 2). Apparently the antibodies bind to the 57 kd polypeptide but do not thereby affect its enzymatic activity. Addition of protein A–Sepharose to samples treated with 57 kd antibodies led to a 30% reduction in the enzyme activity, whereas no reduction was observed in the preimmune control (Figure 5A, columns 3 and 4). Binding of the 57 kd polypeptide–antibody complex to protein A–Sepharose may interfere to a limited degree with the enzyme reaction. When the immune complex was removed from the sample by centrifugation, the supernatant was completely devoid of enzyme activity (Figure 5A, column 6), but the pellet containing the complex also had very little activity (ca. 5% of the total in the extract, column 8). When the pellet and supernatant were mixed again, however, processing activity was reconstituted to almost the original level (Figure 5A, column 9).

The experiment in which gel filtration fractions were mixed (Figure 2) indicated that the 52 kd polypeptide in the supernatants was responsible for activation of the processing enzyme. To substantiate this result, we tested the effect of pure 52 kd polypeptide on the activity of an immunoadsorbed 57 kd component. The purified 52 kd polypeptide led to the same activation as the supernatant fraction (Figure 5B).

The immunoadsorbed 57 kd polypeptide had low but detectable enzyme activity by itself (Figure 5A, column 8). When a 20-fold higher amount of immunoadsorbed 57 kd polypeptide was analyzed in the enzyme assay, the activity was about the same as that of the original sample (Figure 5A, column 10). To control whether this residual activity was due to 57 kd polypeptide alone, we analyzed for possible contamination by 52 kd polypeptide. Immunoblots showed that the anti-57 kd antibody precipitated all the 57 kd polypeptide from mitochondrial extracts and that no 52 kd polypeptide was detectable in the immunoprecipitate (Figure 5C). Titration of the sensitivity of the immunoblot revealed that less than 0.5% of 52 kd polypeptide initially present in the extract could have been detected. On the other hand, about 5% of the initial processing activity was left in the immunoprecipitate of the 57 kd polypeptide (see Figure 5A). The same result was obtained when a preparation of the purified processing enzyme was used in the experiment instead of crude mitochondrial extracts. These data indicate that the 57 kd polypeptide has a low intrinsic enzyme activity in the absence of 52 kd polypeptide and that the latter has a strong stimulating influence on the processing activity.

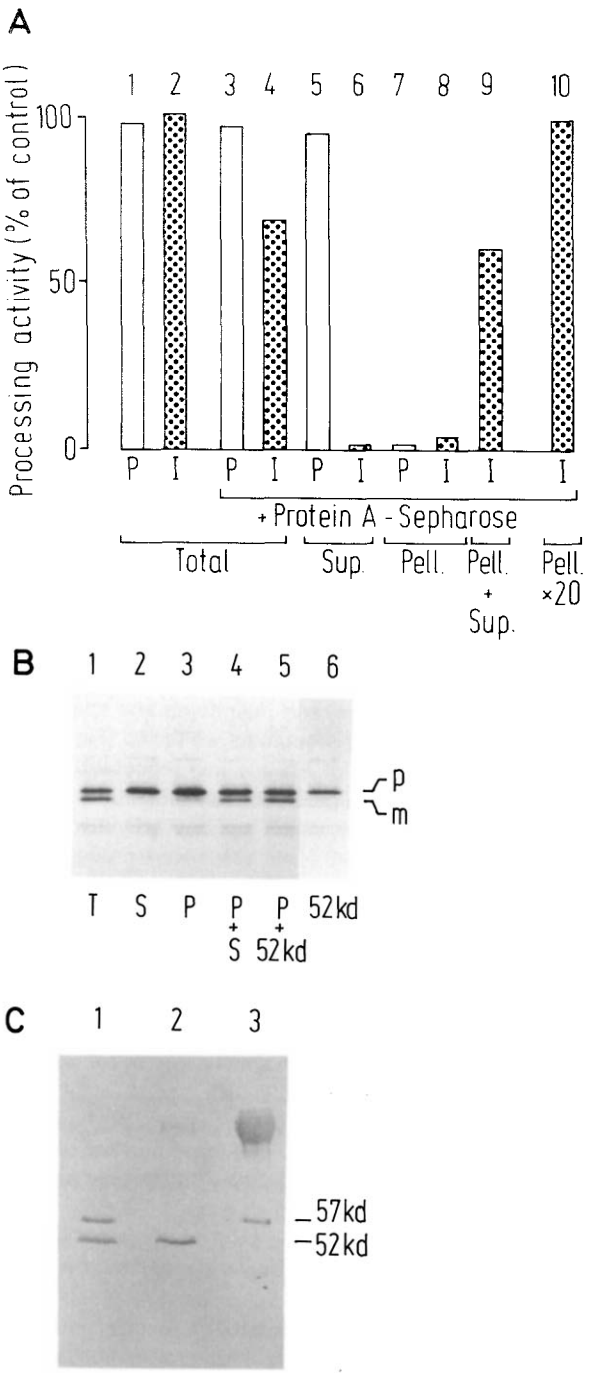

Figure 5. Reconstitution of the Processing Activity by the 52 kd Polypeptide after Isolation of the 57 kd Polypeptide by Immunoadsorption

(A) Mitochondrial pellets (20 μg) were solubilized in a volume of 200 μl as in Figure 3A. Resulting extracts were incubated for 45 min at 4°C with either preimmune globulins or immune globulins directed against the 57 kd polypeptide. Antigen–antibody complexes were then bound to protein A–Sepharose, and the beads were sedimented by centrifugation. Supernatants were kept on ice, and pellets were washed three times with 30 mM Tris–HCl (pH 8.2), 300 mM NaCl, 1% Triton X-100, and resuspended in 200 μl of the same buffer. Aliquots corresponding to 1 μg of untreated mitochondria were taken. Where indicated, supernatants and pellets were remixed and incubated for a further 10 min. All fractions were tested for processing activity. Activities are expressed as a percentage of the activity of the mitochondrial extract without addition of antibodies. Columns 1–4, activities in total fractions; columns 5, 6 and 7, 8, activities in supernatants and pellets, respectively; column 9, activity in pellet and supernatant after remixing; column 10, activity in pellet, but corresponding to 20 μg of mitochondria instead of 1 μg. P, incubation with preimmune globulins. I, incubation with anti-57 kd immune globulins.

(B) Mitochondrial extracts were prepared, and anti-57 kd antibody–protein A–Sepharose complex was added as in (A). Aliquots tested for processing activity corresponded to the extract obtained from 1 μg of mitochondria. Processing of the precursor of ATPase subunit 2 was analyzed. Lane 1, without further treatment (T); lane 2, supernatant (S) after removal of immune complexes by centrifugation; lane 3, pellet (P) containing the 57 kd polypeptide immunoadsorbed to protein A–Sepharose; lane 4, pellet and supernatant remixed; lane 5, pellet after addition of approximately 0.1 μg of 52 kd polypeptide; lane 6, 0.1 μg of 52 kd polypeptide alone. At right, the precursor (p) and mature (m) forms of subunit 2 of F_0F_1-ATPase are indicated.

(C) The 57 kd polypeptide was immunoadsorbed to Sepharose beads as in (A) except that 200 μg of mitochondria was used. One-half of the sample was directly precipitated with TCA. The other half was centrifuged to obtain a pellet, containing the Sepharose beads, and a supernatant; pellet and supernatant were then TCA-precipitated. TCA precipitates were treated with SDS-containing buffer and subjected to electrophoresis. Proteins were blotted to nitrocellulose paper and decorated with antibodies against both 52 kd and 57 kd polypeptides. Bound antibodies were visualized by [^{14}C]protein A and autoradiography. Lane 1, total sample, without separation of immunoadsorbed 57 kd; lane 2, supernatant after removal of protein A–Sepharose beads; lane 3, protein A–Sepharose beads with immunoadsorbed 57 kd.

Part of the 52 kd Polypeptide Is Attached to the Inner Mitochondrial Membrane

Earlier studies localized the processing enzyme as a soluble activity in the mitochondrial matrix (e.g., by digitonin fractionation) (Schmidt et al., 1984). In the isolation procedure employed here, processing activity was extracted by a hypotonic buffer without addition of salt (see Experimental Procedures). On the other hand, we noted that the lower final yield of 52 kd polypeptide, relative to 57 kd polypeptide, was mainly due to losses during this extraction step. We therefore investigated whether the 52 kd component might be partly associated with the inner membrane.

Isolated mitochondria were disrupted by sonication in the presence of increasing salt concentrations, and the release of 52 kd and 57 kd polypeptides into the soluble fraction was measured (Figure 6). At high KCl concentrations up to 85% of the 57 kd polypeptide was extracted from mitochondria, as was the soluble matrix marker fumarase. In contrast, only 20%–30% of the total 52 kd polypeptide was soluble in either the absence or presence of salt. The rest was membrane associated. The integral membrane marker ADP/ATP-carrier was completely recovered in the membrane fraction. This is also known to be the case upon alkaline treatment of mitochondria (Pfanner et al., 1987b). The 52 kd polypeptide, however, was completely extractable from the membranes at alkaline pH (not shown). These data indicate that about 75% of total 52 kd polypeptide is attached to the surface of the inner membrane but in a manner that does not involve direct embedding of the polypeptide in the phospholipid bilayer.

Characteristics of the Purified Processing Enzyme

The final enzyme preparation was analyzed for its catalytic requirement under various conditions. The enzyme

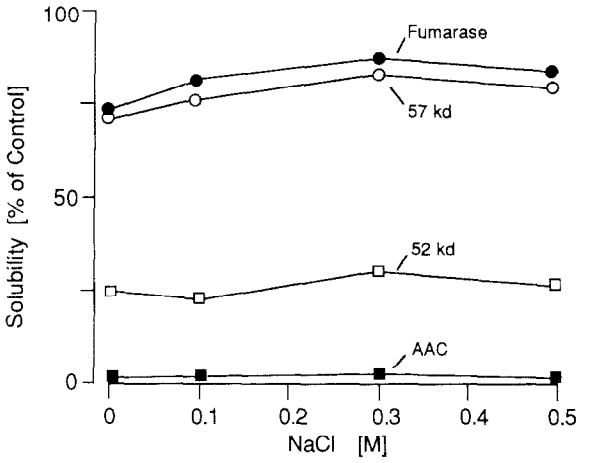

Figure 6. Extraction of 52 kd and 57 kd Polypeptides with Salt

Isolated mitochondria were resuspended in SET buffer (see Experimental Procedures) containing 0–0.5 M NaCl at a protein concentration of 0.1 mg/ml. PMSF and protease inhibitor of N. crassa (Schmidt et al., 1984) were added to final concentrations of 1 mM and 0.1 mg/ml, respectively. Samples were sonicated at 0°C–4°C three times for 1 min using a Branson sonifier with top-end microtip (setting 3, pulsed, 30% duty) and divided into halves. One half was kept on ice for determination of recoveries (total); the other half was centrifuged for 1 hr at 165,000 × g to obtain a membrane and a supernatant fraction. Pellets were resuspended to the same volume as supernatants, and aliquots were taken from all samples for determination of fumarase activities. The rest of each sample was precipitated with TCA. Precipitates were solubilized in SDS-containing buffer, separated by electrophoresis, and then transferred to nitrocellulose paper. Immunodecoration was done with antibodies against 57 kd and 52 kd polypeptides as well as ADP/ATP-carrier (AAC). Bound antibodies were visualized by [^{14}C]protein A and autoradiography. Autoradiographs were quantified by densitometry. Data are expressed as percentages of total immunoreactive protein and enzyme activities, respectively, recovered in the supernatants. Recoveries varied between 84% and 96%.

activity was highly salt sensitive (Figure 7): 60 mM NaCl or KCl was sufficient to reduce the activity by 50%. Removal of salt led to complete restoration of the activity (not shown).

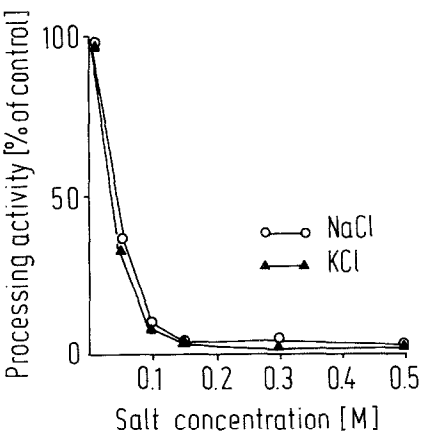

Figure 7. Salt Dependence of Processing Activity

Purified processing enzyme was assayed as described in Experimental Procedures, except that the test buffer was adjusted to different concentrations of either NaCl or KCl. Activities are expressed relative to a control (no salt present).

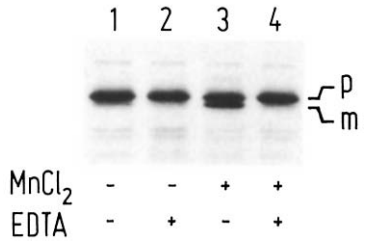

Figure 8. Inhibition of Processing Activity by EDTA

Purified processing enzyme was assayed under different conditions to demonstrate the metal dependence of the enzyme. Lane 1 shows the processing of the precursor of subunit 2 of F_0F_1-ATPase without Mn^{2+} added. In lanes 2 and 4, 5 mM EDTA was included during the test. In lanes 3 and 4, 2 mM $MnCl_2$ was added. Incubation in each case was carried out with 0.15 U of processing enzyme in a total volume of 200 μl.

The purified enzyme was metal dependent. Its activity was enhanced by adding manganese ions (Figure 8, lane 3 vs. lane 1) or zinc or cobalt ions (not shown), and was inhibited by chelators such as EDTA (Figure 8, lanes 2 and 4 vs. lanes 1 and 3).

We investigated whether the purified enzyme cleaved the precursors of various proteins from the mitochondrial matrix, inner membrane, and intermembrane space. Indeed, it cleaved all the precursors we tested (Figure 9): Precursor to subunit 2 of ATPase was processed to the mature-sized form. The precursors of cytochrome c_1 and the Rieske Fe/S protein, which are cleaved in two steps (Teintze et al., 1982; Hartl et al., 1986), were processed to their respective intermediate-sized forms. ATPase subunit 9, which was previously shown to be cleaved in two steps (Schmidt et al., 1984), was processed to the mature-sized form. Furthermore, the precursors of the following proteins (not shown in Figure 9) were found to be cleaved: yeast cytochrome b_2 to the intermediate form; and subunit 3 of ATPase, core proteins I and II of complex III, and isocitrate dehydrogenase to their mature forms. Thus it appears that the purified processing peptidase is responsible for the cleavage of a large number of mitochondrial precursor proteins. It may be the only enzyme that removes matrix-targeting signals.

To demonstrate that we have purified the authentic processing enzyme of mitochondria, we analyzed whether it cleaves at the correct processing site. The precursor to ATPase subunit 9 was translated in a reticulocyte lysate in the presence of [^{35}S]methionine and incubated with the purified enzyme. The mature subunit 9 produced was immunoprecipitated and subjected to radiosequencing. Figure 10 shows that cleavage had occurred at the correct site.

cDNA Cloning and Amino Acid Sequence of the 52 kd Polypeptide

A full-length cDNA clone for the 52 kd polypeptide (insert 108) was selected from a N. crassa cDNA library by antibody screening and colony hybridization (see Experimental Procedures). In vitro expression resulted in the synthesis of an authentic precursor of the 52 kd polypeptide, as revealed by immunoprecipitation and size comparison to

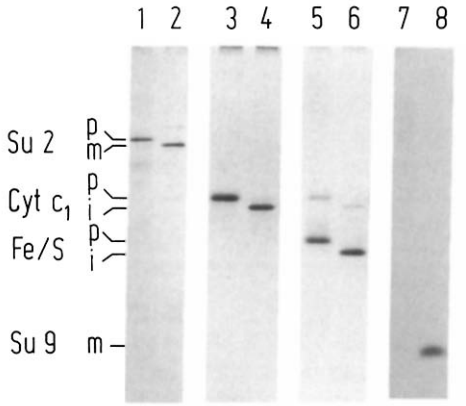

Figure 9. Processing of Different Mitochondrial Precursors by Purified Processing Enzyme

A reticulocyte lysate was programmed with Neurospora crassa poly(A)$^+$ mRNA and translated in the presence of [^{35}S]methionine. The sample was divided into two halves. From one half the various mitochondrial precursor proteins were directly immunoprecipitated (odd lanes). The other half was incubated with the purified processing enzyme, and immunoprecipitation was then performed (even lanes). Lanes 1 and 2 contain ATPase subunit 2; lanes 3 and 4 contain cytochrome c_1; lanes 5 and 6 contain Rieske Fe/S protein; lanes 7 and 8 contain ATPase subunit 9. The precursor (p), intermediate (i), and mature (m) forms of the proteins are labeled.

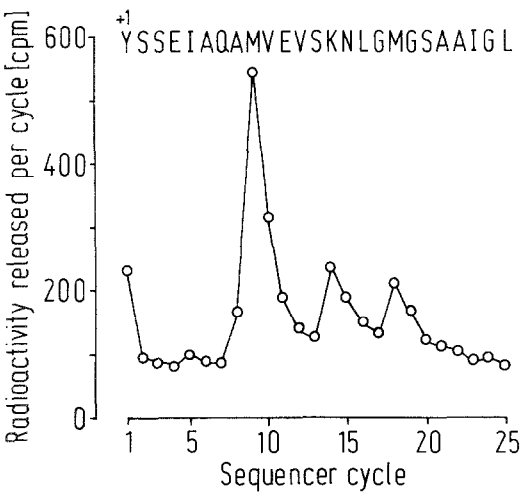

Figure 10. Radiosequencing of Mature ATPase Subunit 9 Produced by Cleavage of the [^{35}S]Methionine-Labeled Precursor with Purified Processing Enzyme

The precursor of ATPase subunit 9 from Neurospora was synthesized in the presence of [^{35}S]methionine from the cloned cDNA by coupled transcription/translation in a reticulocyte lysate (Pfanner et al., 1987a). It was incubated with purified processing enzyme and immunoprecipitated (see Experimental Procedures). The precipitate was subjected to electrophoresis; mature subunit 9 was eluted from the gel (40,000 cpm) and subjected to 25 steps of solid-phase Edman degradation (Wachter et al., 1973). The amino acid sequence of Neurospora subunit 9 is shown (Viebrock et al., 1982); +1 marks the amino terminus of mature subunit 9. Radioactivity is released with methionines at positions 9 and 18, and with lysine at position 14 since subunit 9 is coupled via lysine residues to the solid phase (Schmidt et al., 1984).

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cgctcattccaccccccttcaaatcaccgacctccATGGCTTCCCGGAGACTAGCTCTCAACCTGGCGCAG     36
                                    M  A  S  R  R  L  A  L  N  L  A  Q      12

GGCGTGAAGGCCCGCGCTGGGGGTGTTATCAACCCTTTCCGCAGAGGTCTTGCTACTCCTCACAGCGGCACC    108
 G  V  K  A  R  A  G  G  V  I  N  P  F  R  R  G▲L  A  T  P  H  S  G  T      36

GGCATCAAGACCCAGACCACCACCCTCAAGAATGGCTTGACCGTTGCTTCCCAGTACTCTCCCTACGCCAG    180
 G  I  K  T  Q  T  T  T  L  K  N  G  L  T  V  A  S  Q  Y  S  P  Y  A  Q      60

ACCTCCACGGTTGGCATGTGGATCGATGCCGGTTCCAGGGCCGAGACCGACGAGACCAATGGCACLGCCCAC    252
 T  S  T  V  G  M  W  I  D  A  G  S  R  A  E  T  D  E  T  N  G  T  A  H      84

TTCCTCGAGCACCTTGCCTTCAAGGGAACGACGAAGCGCACTCAGCAACAGTTGGAGCTCGAGATTGAGAAC    324
 F  L  E  H  L  A  F  K  G  T  T  K  R  T  Q  Q  Q  L  E  L  E  I  E  N     108

ATGGGCGCTCACCTCAACGCCTACACCTCGCGCGAGAACACTGTCTACTTTGCCAAGGCTCTCAACGAGGAC    396
 M  G  A  H  L  N  A  Y  T  S  R  E  N  T  V  Y  F  A  K  A  L  N  E  D     132

GTCCCCAAGTGCGTCGACATTCTCCAGGACATTCTTCAGAACTCCAAGCTTGAGGAGTCCGCCATCGAGCGC    468
 V  P  K  C  V  D  I  L  Q  D  I  L  Q  N  S  K  L  E  E  S  A  I  E  R     156

GAGCGCGACGTTATTCTTCGCGAGTCGGAGGAGGTTGAGAAGCAGCTCGAGGAGGTCGTCTTCGACCACCTC    540
 E  R  D  V  I  L  R  E  S  E  E  V  E  K  Q  L  E  E  V  V  F  D  H  L     180

CACGCTACCGCCTACCAGCACCAGCCCCTCGGCCGCACCATCCTCGGTCCCCGTGAGAACATCCGTGACATC    612
 H  A  T  A  Y  Q  H  Q  P  L  G  R  T  I  L  G  P  R  E  N  I  R  D  I     204

ACCCGTACCGAGCTCGTGAACTACATCAAGAACAACTACACCGCCGACCGCATGGTTCTCGTTGGCGCCGGT    684
 T  R  T  E  L  V  N  Y  I  K  N  N  Y  T  A  D  R  M  V  L  V  G  A  G     228

GGCGTCCCCCACGAGCAGCTCGTCGAGATGGCCGACAAGTACTTCTCCAAGCTCCCTGCTACCGCCCCTGTT    756
 G  V  P  H  E  Q  L  V  E  M  A  D  K  Y  F  S  K  L  P  A  T  A  P  V     252

AGCAGCGGTTCCATCCTCTCCAAGAAGAAGCCCGACTTCATCGGCTCTGATATCCGTATCCGGCGACGATACC    828
 S  S  A  S  I  L  S  K  K  K  P  D  F  I  G  S  D  I  R  I  R  D  D  T     276

ATCCCCACCGCCAACATTGCTATTGCCGTTGAGGGTGTCAGCTGGTCCGACGACGACTACTTCACTGGCCTC    900
 I  P  T  A  N  I  A  I  A  V  E  G  V  S  W  S  D  D  D  Y  F  T  G  L     300

GTTACTCAGGCCATCGTTGGCAACTACGACAAGGGTCTTGGCAATGCTCCCCACCAGGGCAGCAAGCTCAGC    972
 V  T  Q  A  I  V  G  N  Y  D  K  A  L  G  N  A  P  H  Q  G  S  K  L  S     324

GGCTTTGTTCACAAGCACGACCTCGCTACCAGCTTCATGAGCTTCTCTACCAGCTATAGCGACACTGGTCTC   1044
 G  F  V  H  K  H  D  L  A  T  S  F  M  S  F  S  T  S  Y  S  D  T  G  L     348

TGGGGTATCTACCTTGTCACTGACAAGCTCGACCGCGTTGACGACCTTGTCCACTTCTCCCTCCGCGAGTGG   1116
 W  G  I  Y  L  V  T  D  K  L  D  R  V  D  D  L  V  H  F  S  L  R  E  W     372

ACCCGTCTCTGCTCCAACGTCAGCGAGGCTGAGGTTGAGCGCCGCAAGGCTCAGCTCAAGGCCTCCATCCTC   1188
 T  R  L  C  S  N  V  S  E  A  E  V  E  R  A  K  A  Q  L  K  A  S  I  L     396

CTCTCCCTTGACGGCACCACCGCCGTTGCTGAGGATATTGGTCGCCAGATCGTCACCACCGGCGCCGCCATG   1260
 L  S  L  D  G  T  T  A  V  A  E  D  I  G  R  Q  I  V  T  T  G  R  R  M     420

AGCCCCGCCGAGATTGAGCGCATCATTGATGCCGTCTCCGCCAAGGACGTTATGGATTTCGCCAACAAGAAG   1332
 S  P  A  E  I  E  R  I  I  D  A  V  S  A  K  D  V  M  D  F  A  N  K  K     444

ATTTGGGATCAGGACATTGCTATCAGCGCGGTCGGCAGCATCGAGGGCCTGTTCGACTATGCCCGTATCAGG   1404
 I  W  D  Q  D  I  A  I  S  A  V  G  S  I  E  G  L  F  D  Y  A  R  I  R     468

GGTGATATGAGCAGGAACGCTTTTtaagcgatagggagcaagaagggggccgggttctgtattataatcggt   1476
 G  D  M  S  R  N  A  F                                                     476

gttacattccagcccaaaatctgtactctagggtcgtaggctagacatagagcaatcaatttccatgcgtac   1548

aaaaaaaaaa                                                                 1558
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Figure 11. Nucleotide Sequence of the 52 kd Protein cDNA and the Deduced Amino Acid Sequence

Nucleotides are numbered starting with the first nucleotide of the first ATG triplet. Amino acids are given in the one-letter code. The proteolytic cleavage site of the processing peptidase is indicated by an arrowhead. The α-helical segment with clustering of negatively charged residues is underline (residues 150–178). The stretches containing only uncharged amino acids are pointed out by dotted lines (residues 47–68 and 332–355, with Asp-345 as an exception). Untranslated regions are in lowercase letters. A putative polyadenylation signal (aatcaa) is underlined.

a 52 kd precursor from a reticulocyte lysate programmed with poly(A)$^+$ mRNA (not shown).

The nucleotide sequence of insert 108 and the deduced amino acid sequence of the precursor of 52 kd polypeptide are given in Figure 11. The coding region of the 52 kd cDNA contains 1428 nucleotides, corresponding to a precursor protein of 476 amino acid residues having a molecular mass of 52,483 daltons. The sequence of the amino terminus of the mature part of the protein was determined by gas-phase sequencing (Lottspeich, 1985) of isolated 52 kd polypeptide (not shown). Threonine, proline, histidine, and serine were detected in positions 3-6, respectively, of the mature-sized protein. Amino acids in positions 1 and 2 could not be identified. Thus the presequence very likely consists of 28 amino acids. It containins 6 basic residues and no acidic residues. The carboxyl terminus of the presequence shows the motif Arg-Arg-Gly fol-

lowed by leucine at position +1 of the mature part, which is similar to the cleavage sites found in a number of other mitochondrial precursor proteins (Schmidt et al., 1984; Hartl et al., 1986; Nicholson and Neupert, 1988). Indeed, the precursor of the 52 kd polypeptide expressed in vitro could be processed to the mature-sized protein by the purified processing enzyme (not shown). Assuming that the precursor form is functionally inactive, the 52 kd polypeptide therefore takes part in its own activation.

The mature protein is largely hydrophilic, with a net charge of −17 (at pH 7). A continuous stretch of moderate hydrophobicity with more than 20 uncharged residues is found near the amino terminus (residues 47 to 68). Another hydrophobic region lies in the carboxy-terminal third of the polypeptide (residues 332 to 355), but is interrupted by a negative charge (aspartic acid) at position 345. These regions are potential candidates for hydrophobic interaction of the 52 kd polypeptide with the inner membrane. With regard to the distribution of charged amino acids, a clustering of 12 acidic residues, interspaced with 4 basic residues, over a stretch of 29 amino acids (residues 150 to 178) is notable. According to secondary structure prediction (Chou and Fasman, 1978), this region has the potential to form an α-helical segment (residues 153 to 178). It might therefore be speculated that such a structural arrangement could be of functional importance for the interaction of the 52 kd polypeptide with the positively charged presequences of imported precursor proteins.

Discussion

During past years a great deal of functional data on the import and assembly of mitochondrial proteins has been collected. We report here the identification of the first two components involved in this complex process.

What are the roles of the 57 kd and 52 kd polypeptides present in the purified preparation of the processing enzyme? Our observations suggest that the peptidase activity is associated with the 57 kd polypeptide and that this component is the matrix processing peptidase (MPP) proper. In the absence of the 52 kd polypeptide, MPP exhibits low but significant activity. In contrast, the 52 kd component alone has no measurable enzyme activity. Because of its stimulating effect, we call the 52 kd component the processing enhancing protein (PEP). PEP is present in large excess with respect to both its content in mitochondria and its activating effect on MPP. Preliminary results suggest that roughly equimolar amounts of PEP are sufficient for full activation.

If MPP performs the actual cleavage reaction, what is the function of PEP? A major task in understanding the mechanism of the processing peptidase is to explain how the enzyme can specifically act on so many different precursors, which differ vastly in the sequence of the cleavage sites and their position within the precursor molecules. Clearly, the information for the site of cleavage must lie in the protein sequence, since alterations of the presequences have been reported to lead to deficiencies in processing (Grivell, 1987; Hurt et al., 1987; Nguyen et al., 1987). For instance, deletion of amino acids 2 to 5 at the amino terminus of a fusion protein between the presequence of cytochrome oxidase subunit IV and mouse dihydrofolate reductase abolishes processing of the precursor by a mitochondrial extract but not targeting of the precursor to isolated mitochondria (Hurt et al., 1985). Our working hypothesis is that by the cooperation of two different proteins in the processing reaction, which may recognize different parts of the presequences, the reaction is greatly enhanced in rate and perhaps specificity. For example, one could imagine that PEP recognizes a certain general structural determinant of the presequences (e.g., amphipathic α-helix or β-sheet) and, by binding to it, presents the precursor to the actual proteolytic component. With this assisting function, PEP might increase the catalytic rate and the specificity by a considerable factor. Indeed, we have never observed that the purified enzyme makes any further proteolytic cleavage besides removing the presequence.

Besides its participation in the cleavage of presequences, PEP could have additional functions important for the transfer of proteins into mitochondria. PEP localized at the inner surface of the inner membrane could interact with the presequences of imported precursor proteins as soon as they enter the matrix space, thus playing a role in membrane translocation or preventing an unfavorable misfolding of the precursor. It is possible that the membrane-bound and soluble forms of PEP represent different functional states of the polypeptide.

Recently, temperature-sensitive mutations in Saccharomyces cereviseae were described that affect the processing of mitochondrial precursor proteins (Yaffe and Schatz, 1984; Yaffe et al., 1985). Two complementation groups were identified (*mas-1* and *mas-2*) in which precursor proteins accumulated at the restrictive temperature. Matrix extracts from *mas-1* cells have strongly reduced processing activity. An antibody prepared against a fusion protein between the amino-terminal fragment of the *mas-1* gene product and β-galactosidase specifically cross-reacted in Western blots with PEP of Neurospora mitochondrial extracts and with the purified processing enzyme. Furthermore, the protein sequence of PEP shows 60% homology to that of the *mas-1* gene product (Witte et al., 1988). This observation suggests that *mas-1* encodes the yeast equivalent to the Neurospora PEP. Interestingly, *mas-1* cells at the nonpermissive temperature have been reported to accumulate precursors outside mitochondria (Yaffe and Schatz, 1984). This may support the idea of a role of PEP in the translocation of precursors into the mitochondria, as discussed above, in addition to its role in enhancing precursor processing.

The mitochondrial processing peptidase represents a new type of signal-cleaving enzyme that is different in almost all respects from other peptidases involved in protein translocation—in particular, the well-characterized bacterial leader peptidase (Zwizinski and Wickner, 1980; Wolfe et al., 1983) and the eukaryotic signal peptidase of the endoplasmic reticulum (Evans et al., 1986). The latter two enzymes are related with respect to their ability to correctly process both prokaryotic and eukaryotic signal sequences (Watts et al., 1983). Furthermore, both the leader

peptidase and signal peptidase are membrane-integrated enzymes. Bacterial leader peptidase consists of a single integral membrane polypeptide, whereas the signal peptidase has been suggested to be present in a large membrane complex and may constitute part of a proteinaceous pore of the translocation apparatus (Evans et al., 1986). In contrast, the mitochondrial processing enzyme is not integrated in the membrane, and it does not cleave precursors of secreted proteins. Also, in contrast to leader and signal peptidase, it is a metal-dependent enzyme. Finally, two polypeptides are required for activity, and these are present as monomers and do not form a stoichiometric complex.

Thus the mitochondrial MPP appears to represent a member of a new class of processing enzymes. The corresponding enzyme in the chloroplast stroma may also belong to this new class. It is not particularly surprising that in organelles such as mitochondria and chloroplasts, the processing enzymes are not related to those cleaving secreted precursors. The general mechanisms and pathways of importing precursor proteins into organelles bounded by a double membrane are grossly different from the translocation of precursors across the bacterial plasma membrane or across the membrane of the endoplasmic reticulum. In earlier studies on the complex sorting pathways of precursors into the mitochondrial intermembrane space, we have described that this process involves complete transfer into the matrix and retranslocation across the inner membrane (Hartl et al., 1987). It is tempting to propose that the processing enzyme(s) participating in the latter step, namely, cleavage of the second part of the presequence from the respective precursors, bears evolutionary relationships to the bacterial leader peptidase.

Experimental Procedures

Growth of Neurospora and Preparation of Mitochondria

N. crassa wild-type strain 74A was grown for 15 hr as described by Korb and Neupert (1978). Cells were harvested by filtration. Storage at −20°C for several months did not lead to loss of processing peptidase activity. Mitochondria were isolated as published (Korb and Neupert, 1978) in SET buffer (0.25 M sucrose, 1 mM EDTA, 30 mM Tris–HCl [pH 7.5]) containing 1 mM PMSF. They were washed once in the same buffer without EDTA, sedimented by centrifugation for 15 min at 14,000 × g, and stored at −20°C.

Purification of the Processing Enzyme

All the following steps were carried out at 4°C. A preparation routinely started with 200 g of frozen cells.

Preparation of a Total Extract

Neurospora hyphae were resuspended in 1200 ml of isolation buffer (30 mM Tris [pH 8.2], 0.1 mM PMSF) using a Waring blender. The cells were then broken by passing them three times through a grind mill (Sebald et al., 1979). Cellular debris and unbroken cells were removed by centrifugation for 2 hr at 17,700 × g. The resulting supernatant was adjusted to pH 7.5 with concentrated HCl. It was referred to as total extract.

DEAE-Cellulose Chromatography

The total extract was loaded onto a DE-52 column (Whatman) (2.5 × 35 cm) equilibrated with 10 mM Tris–HCl (pH 7.5). After washing with 1.4 l of equilibration buffer proteins were eluted with 1.7 l of a salt gradient from 0 to 300 mM NaCl in equilibration buffer (flow rate 3.3 ml/min). The processing activities of different fractions were determined according to the standard processing peptidase assay. The active fractions, which eluted at 200 mM NaCl, were pooled.

Zn-Chelate Chromatography

Metal-chelate-affinity Sepharose 6B (Pharmacia) (100 ml) was loaded with Zn^{2+} ions to saturation. The column (2.5 × 20 cm) was equilibrated with 30 mM Tris–PO_4 (pH 7.0), 200 mM NaCl. The pooled activity from the previous column was loaded. Washing was done with 800 ml of equilibration buffer. Elution was carried out with a linear gradient (1 l) from 0 to 30 mM histidine in equilibration buffer (flow rate 2.5 ml/min). Active fractions were pooled.

PEI-Cellulose Chromatography

PEI-cellulose (Sigma) (2.5 × 10 cm) was equilibrated with 10 mM Tris–PO_4 (pH 7.0), 100 mM NaCl. The processing peptidase pool of the Zn-chelate affinity eluate was diluted 2-fold with H_2O and applied to PEI-cellulose. The column was washed with 250 ml of equilibration buffer and eluted with a 500 ml gradient from 10 to 100 mM Tris–PO_4 (pH 7.0), 100 mM NaCl (flow rate 2.5 ml/min). Aliquots were taken for determination of processing peptidase activity, and the remaining fractions were immediately subjected to precipitation with PEG.

PEG Precipitation

PEG precipitation was performed according to Ingham (1984). A stock solution of 28% (wt/vol) of PEG 8000 (Sigma) in H_2O was prepared. To each of the eluted fractions of the PEI-column an equal volume of the PEG stock solution was slowly added and gently stirred overnight. Active fractions were pooled and centrifuged for 20 min at 16,000 × g. The pellet was washed once with 14% PEG and resuspended in 10 ml of 10 mM Tris–HCl (pH 7.5), 1% Triton X-100.

Ecteola-Cellulose Chromatography

The resuspended pellet was adjusted to 300 mM NaCl by adding solid NaCl. After stirring for 30 min, virtually all of the protein was resolubilized. The solution was cleared by centrifugation for 10 min at 16,000 × g and the supernatant was diluted with 30 mM Tris–PO_4 (pH 7.0), 1% Triton X-100 to a final NaCl concentration of 75 mM (volume 40 ml). It was then loaded onto a column of Ecteola-cellulose (Sigma) (0.6 × 7.0 cm) equilibrated with 10 mM Tris–PO_4 (pH 7.0), 75 mM NaCl, 1% Triton X-100. After washing with 15 ml of the same buffer, processing activity was eluted with a linear salt gradient from 75 to 300 mM NaCl in equilibration buffer (flow rate 0.17 ml/min). All eluted fractions were precipitated with PEG by adding an equal volume of a 40% stock solution and gently mixing end over end for 24 hr. Precipitates were resolubilized in 10 mM Tris (pH 7.5). Active fractions were pooled and stored at −20°C. To obtain purified 52 kd polypeptide, fractions eluting at a salt concentration of 100–120 mM were pooled and PEG-precipitated as described above. These fractions were inactive in the processing assay and contained only 52 kd polypeptide as revealed by SDS–polyacrylamide gel electrophoresis and Western blotting.

Superose 12 Gel Chromatography

FPLC gel chromatography was performed on a Superose 12 column (Pharmacia) equilibrated with 10 mM Tris–HCl (pH 7.5), 200 mM NaCl. The eluate of Zn-chelate chromatography (0.5 ml) containing 0.8 mg of protein was injected. The column was developed at a flow rate of 0.5 ml/min. No processing activity appeared in the first 12 ml of eluate. Fractions of 0.25 ml were collected and stored at −20°C.

Preparation of Specific Antisera

Antisera were raised in rabbits according to Knudsen (1985), with some modifications. Purified processing enzyme from a routine preparation was electrophoresed on an SDS–polyacrylamide gel and blotted to nitrocellulose paper (Burnette, 1981). The 52 kd and 57 kd polypeptides were stained with Ponceau S, and the bands were cut out and dried. After solubilization of the nitrocellulose-bound proteins in dimethyl sulfoxide (0.3 ml) and addition of an equal volume of Freund's complete adjuvant (first injection) or incomplete adjuvant (all subsequent injections), the mixture was injected subcutaneously in the neck region twice at biweekly intervals. One month after the second injection the rabbits were bled, and the presence of antibodies was checked by Western blotting. Immunoglobulins were prepared by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (Deutsch, 1967; Fahey, 1967).

Synthesis of Precursor Proteins

Cell-free synthesis of N. crassa precursor proteins in rabbit reticulocyte lysates (Pelham and Jackson, 1976) was carried out as described before using either poly(A)$^+$ mRNA (Schleyer et al., 1982) or mRNA produced by in vitro transcription from cDNA cloned into transcription

vectors (Pfanner et al., 1987a; Hartl et al., 1987; Kleene et al., 1987). Desalting of postribosomal supernatants was performed by gel filtration using PD-10 columns (Pharmacia).

Processing Peptidase Assay

One hundred microliters of desalted lysate containing radiolabeled N. crassa precursor proteins was mixed with the processing enzyme fraction to be tested. When necessary, 30 mM Tris–HCl (pH 8.2) was added to keep the NaCl concentration in the final assay below 30 mM. Ten microliters of 10% Triton X-100, 1 μl of 0.1 M PMSF (in ethanol), and 2 μl of 0.1 M $MnCl_2$ were added to a 100 μl assay, and incubation was carried out for 60 min at 25°C. In cases where the enzyme was bound to protein A–Sepharose via specific antibodies, all samples were shaken end over end during incubation. The processing reaction was stopped by addition of NaCl and EDTA from 10-fold concentrated stock solutions to final concentrations of 300 mM and 5 mM, respectively. Processing products were analyzed by immunoprecipitation (Schleyer and Neupert, 1985), SDS–polyacrylamide gel electrophoresis, and fluorography.

One unit of enzyme activity is defined as the amount of enzyme that processes 1 fmol/min of the precursor of ATPase subunit 2 under the conditions described above.

Quantification of the Amount of Precursor of ATPase Subunit 2 Synthesized in Reticulocyte Lysates

Proteins were synthesized in rabbit reticulocyte lysates in the presence of N. crassa poly(A)$^+$ mRNA, and radiolabeled with [^{35}S]methionine as described above. The precursor of ATPase subunit 2 was quantitatively immunoprecipitated from each lysate used and subjected to SDS–polyacrylamide gel electrophoresis and fluorography. The radioactive bands of three parallel precipitations were cut out, and the radioactivity was determined according to Bonner (1983). Based on the specific radioactivity of methionine in the lysate and the number of methionines in precursor of ATPase subunit 2, the concentration of precursor was calculated to be 57 pM, with only slight variation between different lysates.

cDNA Cloning and DNA Sequencing

Antibody screening of a N. crassa cDNA library (Kleene et al., 1987) in pEX vectors for 52 kd polypeptide clones was performed according to Stanley and Luzio (1984). One short cDNA insert, which was also positive in hybrid selection (Viebrock et al., 1982) of 52 kd mRNA, was used to screen a N. crassa cDNA library in pBR322 by colony hybridization. Two positive clones were obtained (inserts 107 and 108; length of the inserts ca. 1.6 kb). For in vitro expression, cDNA insert 108 was cut with NcoI (deleting the entire 5′ noncoding region including the dG-dC tails introduced by the cDNA cloning procedure) and cloned into transcription vector pGEM3 (Promega) (Stuart et al., 1987). For supercoil sequencing (Chen and Seeburg, 1985), full-length cDNA insert 108 was cloned into pUC19 in both orientations and shortened by the ExoIII method (Henikoff, 1984). Parts of the cDNA clones and of genomic 52 kd clones were also sequenced using 52 kd–specific DNA primers.

Miscellaneous

Fractionation by sonication and alkaline treatment of mitochondria were carried out exactly as described previously (Hartl et al., 1987; Fujiki et al., 1982; Pfanner et al., 1987b). TCA precipitation of proteins and determination of protein concentration were performed according to published procedures (Bradford, 1976; Bensadoun and Weinstein, 1976). SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). For fluorography, gels were incubated for 30 min in Amplify (Amersham) and were exposed to Kodak X-Omat AR films at −80°C. Metabolically labeled mitochondria were isolated from cells grown in the presence of [^{35}S]sulfate or [^{3}H]leucine (Korb and Neupert, 1978; Hartl et al., 1986).

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