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Processing peptidase of *Neurospora* mitochondria

Two-step cleavage of imported ATPase subunit 9

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Subunit 9 (dicyclohexylcarbodiimide binding protein, 'proteolipid') of the mitochondrial F_1F_0-ATPase is a nuclearly coded protein in *Neurospora crassa*. It is synthesized on free cytoplasmic ribosomes as a larger precursor with an NH_2-terminal peptide extension. The peptide extension is cleaved off after transport of the protein into the mitochondria. A processing activity referred to as processing peptidase that cleaves the precursor to subunit 9 and other mitochondrial proteins is described and characterized using a cell-free system. Precursor synthesized *in vitro* was incubated with extracts of mitochondria. Processing peptidase required Mn^{2+} for its activity. Localization studies suggested that it is a soluble component of the mitochondrial matrix. The precursor was cleaved in two sequential steps via an intermediate-sized polypeptide. The intermediate form in the processing of subunit 9 was also seen *in vivo* and upon import of the precursor into isolated mitochondria *in vitro*. The two cleavage sites in the precursor molecule were determined. The data indicate that: (a) the correct NH_2-terminus of the mature protein was generated, (b) the NH_2-terminal amino acid of the intermediate-sized polypeptide is isoleucine in position −31. The cleavage sites show similarity of primary structure. It is concluded that processing peptidase removes the peptide extension from the precursor to subunit 9 (and probably other precursors) after translocation of these polypeptides (or the NH_2-terminal part of these polypeptides) into the matrix space of mitochondria.

The majority of mitochondrial proteins are encoded by nuclear genes [1]. They are synthesized on free cytoplasmic ribosomes as water-soluble species, i.e. precursor proteins [2]. In many cases these precursor forms carry NH_2-terminal peptide extensions not present in the mature proteins [3]. The free precursors are then selectively transported to their functional sites within the mitochondria. Transport is accompanied by one or more processing steps which may include covalent modifications of precursor proteins and/or proteolytic removal of peptide extensions. The assembly of cytochromes *c* and c_1 requires the covalent attachment of the heme group [4 – 6]. In a few cases proteolytic processing takes place in two steps, as has been shown for cytochrome c_1 in *Neurospora* and yeast and for cytochrome b_2 in yeast [5, 6].

The mitochondrial enzymes involved in processing have not been extensively characterized. Proteolytic activities have been detected in extracts of mitochondria from different sources [7 – 9], which can remove the NH_2-terminal peptide extensions or at least part of them from precursor proteins. A partial purification of this activity from yeast mitochondria has been achieved [10]. The processing activities investigated so far have several features in common: they appear to be soluble components of the mitochondrial matrix and they require divalent metal ions such as Mn^{2+} or Zn^{2+} for full activity. The processing activity responsible for the second cleavage of cytochromes c_1 and b_2 is thought to reside on the outer face of the inner mitochondrial membrane [6]. This latter activity does not require divalent metal ions, but a submitochondrial processing assay has not yet been established.

We have characterized the processing activity referred to as processing peptidase with cleaves the precursor to *Neurospora* ATPase subunit 9 ('proteolipid', dicyclohexylcarbodiimide binding protein, Su 9). The precursor (pre Su 9) has an M_r of 16400 whereas the mature protein has an M_r of 10500 [11]. We have used a cell-free translation system as a source for the precursor, and extracts from isolated *Neurospora* mitochondria as a source of processing peptidase. The activity depends on the presence of divalent metal ions and can be inhibited by chelating agents. It appears to be located in the mitochondrial matrix and cleaves pre Su 9 in two steps via an intermediate-sized polypeptide. The two cleavage sites show striking similarities in their primary structure as deduced from radiosequencing experiments. The results presented suggest that the intermediate-sized polypeptide represents a true intermediate in the assembly pathway of Su 9.

MATERIALS AND METHODS

Growth of Neurospora *and preparation of mitochondria*

Neurospora crassa wild-type 74A was grown and metabolically labeled as described [12]. Mitochondria were isolated after disrupting cells by grinding with sand according to published procedures [12]. The isolation medium was 0.25 M sucrose, 1 mM EDTA, 30 mM Tris/HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride.

Subfractionation of mitochondria

For preparation of mitochondrial extracts, mitochondria were suspended at a concentration of 5 mg mitochondrial

protein/ml in 30 mM Tris/HCl, pH 8.2, 0.1 mM phenylmethylsulfonyl fluoride. The suspension was sonicated 3 × 5 s at 1-min intervals and centrifuged for 1 h at 166000 × *g*. The supernatant containing the processing activity was stored in aliquots at −20 °C.

For localization experiments, mitochondria were fractionated as follows: mitochondria were suspended at a concentration of 7 mg mitochondrial protein/ml in 0.25 M sucrose, 30 mM Tris/HCl, pH 7.5, 0.1 mM phenylmethylsulfonyl fluoride (STP buffer) and incubated for 10 min at 0 °C with 1.4 mg/ml lyophilized cytosolic proteinase inhibitor fraction (see below). 5 100 µl aliquots of this suspension were separately mixed with 100 µl of a freshly prepared solution of digitonin (Merck, Darmstadt, FRG) (0−0.8% w/v in STP buffer, made up from the same stock), so that the final concentrations were 0, 0.1, 0.2, 0.3, 0.4% of digitonin, respectively. After 1 min of incubation at 0 °C, the samples were diluted by adding 1.2 ml of STP buffer and layered on a sucrose cushion of 600 µl of 0.7 M sucrose, 30 mM Tris/HCl, pH 7.5. The samples were centrifuged for 10 min at 48000 × *g*. The supernatants including the sucrose cushion (2 ml total) were removed and the pellets were resuspended in 200 µl of STP buffer. Aliquots of the fractions corresponding to 35 µg of untreated mitochondria were immediately assayed for processing activity and the rest of the samples was stored at −20 °C.

Synthesis of precursor proteins

Cell-free synthesis of *Neurospora* precursor proteins in rabbit reticulocyte lysates was carried out as before [13]. Postribosomal supernatants of lysates were desalted by centrifuging through Sephadex G-25 equilibrated with 30 mM Tris/HCl, pH 8.2 and stored in aliquots of 100 µl at −75 °C.

For sequencing experiments the precursor to Su 9 was synthesized *in vitro* in a total volume of 20−60 µl of translation mixture (reticulocyte lysates, Amersham Buchler, Braunschweig, FRG) in the presence of 1.2 mCi/ml [^{35}S]methionine (1250 Ci/mmol, Amersham) or 1 mCi/ml [^{3}H]leucine (110 Ci/mmol, NEN Chem., Boston, MA, USA). The lysates were programmed with hybridization-selected mRNA specific for Su 9 [14].

Transfer in vivo *of subunit 9*

Neurospora cells were grown for 14 h as described above. An aliquot of the culture containing 30 mg of cells was removed and cooled to 8 °C. Cells were labeled with 5 µCi/ml [^{3}H]leucine (50 Ci/mmol, NEN Chem., Boston, MA) for 3 min, and the assay was divided into three portions. The first portion was immediately precipitated by adding trichloroacetic acid to a final concentration of 0.3 M. The remaining two portions were subjected to another incubation for 3 min at 8 °C in the presence of 0.1 mg/ml cycloheximide or cycloheximide and EDTA/1,10-phenanthroline (5 mM/1 mM, respectively). Then these portions were also precipitated with trichloroacetic acid. The samples were kept at 0 °C for 30 min, washed with 5 ml of acetone and dissolved in 1 ml of 1% (w/v) sodium dodecylsulfate, 50 mM NaP_i, pH 8.0 by boiling for 5 min. After dilution with Triton containing buffer (1% w/v Triton X-100, 0.3 M NaCl, 5 mM EDTA, 10 mM Tris/HCl, pH 7.5) the samples were immunoprecipitated for Su 9 as described previously [11].

Isolation of proteins and immunoprecipitation

Cytosolic proteinase inhibitor fraction was prepared according to the following protocol: 150 g of freshly harvested *Neurospora* cells were washed in 6 l of ice-cold water. Cells were homogenized in a Waring blendor after adding 450 ml of water. The slurry was made 5% (v/v) in perchlorid acid and incubated for 10 min at 65 °C. After cooling to room temperature, 5 M KOH was added until neutral pH was reached. The mixture was centrifuged for 10 min at 12000 × *g*. The supernatant was subjected to fractionation by ammonium sulphate precipitation. Material that precipitated between 25% and 75% saturation was collected and dissolved in water. After a clarifying spin (20 min at 29000 × *g*) the solution was vigorously shaken for 5 min with an equal volume of chloroform. The aqueous phase was recovered by centrifugation and lyophilized. The dried material was redissolved in 10 ml of water and subjected to gel filtration on a Sephadex G-25 column (25 × 350 mm) equilibrated with water. The flow rate was 120 ml/h. Turbid fractions appearing in the void volume were pooled, lyophilized and redissolved in water at a protein concentration of 1 mg/ml and stored at −20 °C. The yield was 250 µg/g of hyphae. This fraction was able to inhibit mitochondria associated proteolytic activity measured with azocoll as a substrate by 90% at a ratio of 140 µg/mg mitochondrial protein.

Isolation of proteins, preparations of antibodies and immunoprecipitation of Su 9 [11, 15], F_1ATPase subunit β [16], Fe-S protein [5], cytochrome c_1 [5], porin [17], and citrate synthase [18] were performed as described earlier.

Determination of enzymatic activities

Fumarase and succinate-cytochrome-*c*-reductase activities were determined according to [19, 20]. Adenylate kinase was assayed in a mixture composed of 1 ml of 130 mM KCl, 6 mM $MgSO_4$, 100 mM Tris/HCl, pH 7.5, 20 µl of 0.1 M NADH, 5 µl of 0.1 M ATP, 10 µl of 50 mM phospho*enol* pyruvate, 5 µl of 1 mM rotenone, 5 µl of 1.5 mM oligomycin, 10 µl of a mixture of pyruvate kinase and lactate dehydrogenase (20 U/ml each), 200 µl of the sample. The reaction was started by adding 5 µl of 0.15 M AMP and followed by the decrease of absorbance at 366 nm. The samples for fumarase and adenylate kinase assays were adjusted to 2.5% (w/v) Genapol X-100 (Hoechst AG, Frankfurt, FRG) immediately before starting the reaction. The processing activity was determined as follows: 100 µl of desalted reticulocyte lysate containing radiolabeled precursor proteins were mixed with 100−400 µl containing the sample to be assayed and 30 mM Tris/HCl, pH 8.2. Then 10 µl of 10% (w/v) Triton X-100, 1 µl of 0.1 M phenylmethylsulfonyl fluoride (in ethanol) and 2 µl of 0.1 M $MnCl_2$ were added per 100 µl of assay volume. The mixture was incubated for 1 h at 25 °C. The reaction was stopped by adding 10 µl of 3 M NaCl, 50 mM EDTA, pH 8.2 per 100 µl of volume. The processing products were analyzed by immunoprecipitation, dodecylsulfate polyacrylamide electrophoresis and fluorography (see below).

Analytical procedures

Protein was determined according to Bradford [21]. Electrophoresis in 16% polyacrylamide gels was carried out according to Laemmli [22]. Fluorography and densitometry of films were performed as previously [23, 24]. Isolation of

proteins for Edman degradation was carried out by immunoprecipitation and subsequent electrophoresis. After fluorography the bands of interest were cut out, washed in water and extracted with 600 µl of 1% (w/v) SDS, 30 mM NaP_i, pH 8.2 at 57°C overnight. The eluted material was subjected to automated solid phase Edman degradation [25]. Immunoreplica analysis using lactoperoxidase linked to sheep anti-rabbit-antibodies was performed as described in [26].

RESULTS

Processing peptidase cleaves the precursor to ATPase subunit 9 in two steps

Radiolabeled reticulocyte lysates containing pre Su 9 were incubated with a fixed amount of an extract prepared from *Neurospora* mitochondria. The processing products were analyzed by immunoprecipitating the samples with antibodies against Su 9. The time course of processing over a period of 1.5 h is shown in Fig. 1A. The appearance of mature-sized Su 9 (Fig. 1A, lane 10) is preceeded by the formation of a band which has an M_r of 13000. This intermediate was also observed, when intact *Neurospora* mitochondria were incubated with radiolabeled reticulocyte lysates in the presence of chelating agents to inhibit processing peptidase [16]. The processing activity observed in this experiment is clearly contributed by the mitochondrial extract, because upon incubation without extract there was no detectable processing (Fig. 1A, lane 9). As mentioned earlier [11], the antibody against Su 9 does not precipitate the precursor very efficiently, apparently because the large additional sequence consisting of 66 essentially hydrophilic amino acids [14] shields or alters antibody binding sites on the 81 amino acid sequence corresponding to the mature protein. The time course shown in Fig. 1A was quantified by densitometry of exposed films (Fig. 1B) and compared to a dose-response curve that was processed in the same way (Fig. 1C). The last point in the dose-response curve showed a band pattern comparable to that of lane 5 in Fig. 1A. Both curves gave essentially a linear response up to a point where approximately equal amounts of intermediate-sized and mature-sized Su 9 were produced. So it is possible to regard them a standard curves of enzymatic activity to which any given activity can be compared and quantified irrespective of whether one calculates the ratio m/i (m = amount of mature Su 9, i = amount of intermediate) or the sum of processing products $m + i$.

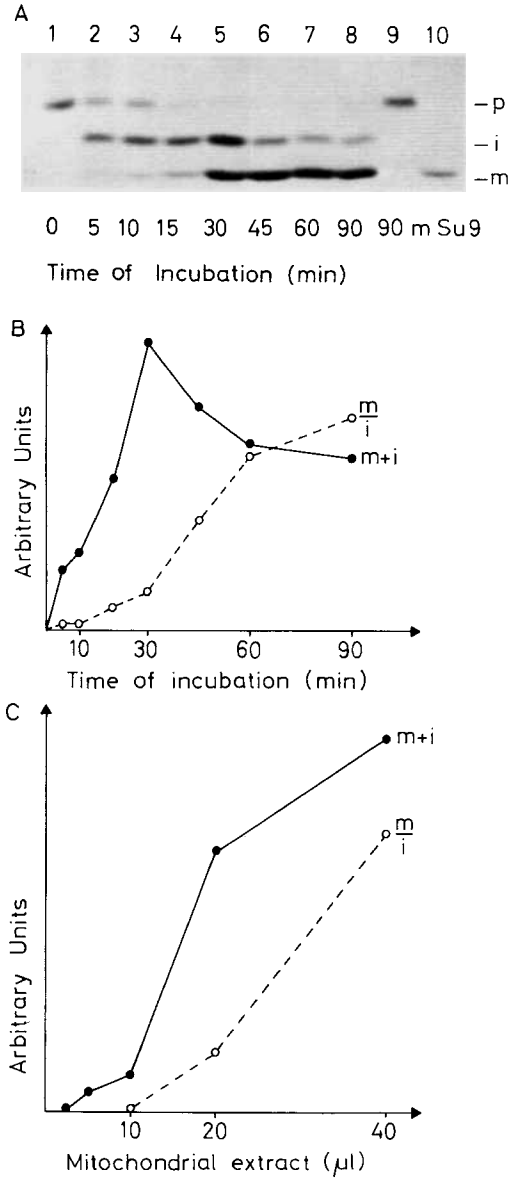

Fig. 1. *Processing of the precursor to subunit 9 by a mitochondrial extract.* (A) Aliquots of desalted lysate (100 µl each) containing radiolabeled precursors were incubated as described in Materials and Methods in a final volume of 225 µl with 20 µl of a mitochondrial extract for various time periods. Su 9 was immunoprecipitated and subjected to electrophoresis. Bands were visualized by fluorography. Lane 9 contained no mitochondrial extract. Mature Su 9 (lane 10) was immunoprecipitated from mitochondria labeled *in vitro*. p, precursor; i, intermediate; m, mature Su 9 (B). The time course of processing shown in A was quantified by densitometry of exposed films. The values of $m + i$ and m/i are plotted versus the time of incubation. (C) Aliquots of desalted lysate (100 µl each) were incubated as in A with the indicated amounts of mitochondrial extract for 1 h. Su 9 was immunoprecipitated and subjected to electrophoresis. Bands were quantified by densitometry of exposed films. The values of $m + i$ and m/i are plotted versus the volume of mitochondrial extract

Processing peptidase activity depends on the presence of Mn^{2+}

Processing activities in extracts of mitochondria were shown to depend on the presence of divalent metal ions such as Zn^{2+} or Mn^{2+} [7, 8]. This is also true for *Neurospora* processing peptidase (Fig. 2, lane 2 versus lane 3). Accordingly, processing peptidase is inhibited by chelating agents such as phenanthrolines and EDTA (Fig. 2, lanes 4 – 6). This experiment also shows that not only the first step is metal dependent (Fig. 2, lanes 4, 6), but also the second step (Fig. 2, lane 2). If the second step does not require metal ions, one would except to see only mature Su 9 in Fig. 2, lane 2. The Mn^{2+}-concentration in this sample was suboptimal.

The inhibition of processing peptidase by chelating agents was also observed *in vivo*. *Neurospora* cells were pulse labeled with [^{3}H]leucine and subjected to a chase period in the presence and absence of EDTA and 1,10-phenanthroline. As shown in Fig. 3, the chelating agents inhibited the production of mature Su 9 and conserved the band pattern obtained during the pulse showing precursor, intermediate, and mature Su 9. The conditions employed were not such as to inhibit transport of proteins into mitochondria. Transport of ADP/

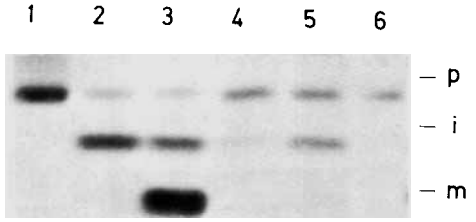

Fig. 2. *Processing peptidase is inhibited by chelating agents.* Aliquots of desalted lysate containing radiolabeled precursors (100 µl each) were incubated with 20 µl of mitochondrial extract in a final volume of 225 µl for 1 h. Reactions contained: lanes 2, 3, no additions; lane 4, 2 mM 1,10 phenanthroline; lane 5, 2 mM bathophenanthroline disulfonic acid; lane 6, 2 mM EDTA. In lane 2, Mn^{2+}, usually present in the processing assay at a concentration of 2 mM (see Materials and Methods), was omitted. Lane 1 shows precursor immunoprecipitated from untreated lysate. Su 9 was immunoprecipitated and subjected to electrophoresis. Bands were visualized by fluorography. p, precursor; i, intermediate; m, mature Su 9

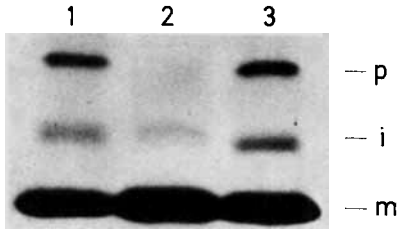

Fig. 3. *Processing* in vivo *of subunit 9 is inhibited by chelating agents.* 30 mg of *Neurospora* cells (wet weight, 10 ml of culture) were pulse labeled with 50 µCi of [^{3}H]leucine for 3 min at 8 °C. The culture was divided into three portions. The first one was immediately precipitated with trichloroacetic acid (final concentration 0.3 M) (lane 1). The remaining two portions were further incubated for 3 min at 8 °C in the presence of 0.1 mg/ml cycloheximide (lanes 2, 3) and 5 mM EDTA/1 mM 1,10-phenanthroline (lane 3 only). Then these protions were precipitated with trichloroacetic acid. After solubilization of proteins, Su 9 was immunoprecipitated and subjected to electrophoresis. Bands were visualized by fluorography. p, precursor; i, intermediate; m, mature Su 9

ATP carrier, a protein whose precursor has no cleavable peptide extension [27] was not inhibited (data not shown), which indicates that transfer of proteins into mitochondria is not unspecifically affected. This result further suggests that intermediate sized Su 9 is not an *in vitro* artifact.

Processing peptidase could not be inhibited by *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, benzamidine, phenylmethylsulfonyl fluoride, 1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), *N*-tosyl-lysine chloromethyl ketone (TLCK), aprotinin, bestatin or EGTA (data not shown). This is in agreement with the behaviour of mitochondrial processing enzymes described by others [8, 10]. Another characteristic of the peptidase is that the activity in this assay is stimulated about three fold by Triton X-100 at a concentration of 1% (w/v) (data not shown). An explanation for this observation might be that the detergent mimics a membranous environment for the precursor thus facilitating the action of processing peptidase. It was suggested previously [16] that pre Su 9 embedded in the inner membrane of mitochondria is the substrate for processing peptidase in intact mitochondria.

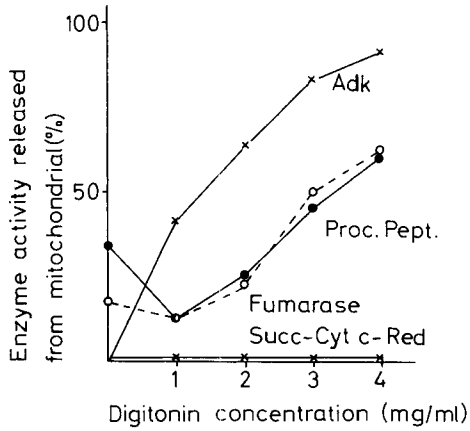

Fig. 4. *Localization of processing peptidase.* Mitochondria were treated with digitonin as described in Materials and Methods and recovered by centrifugation. Aliquots of the resuspended mitochondria and the respective supernatants corresponding to 35 µg of untreated mitochondria were assayed for processing activity in the presence of Triton X-100. The assay was carried out in a final volume of 600 µl for 1 h employing 100 µl of desalted lysate containing radiolabeled precursors. Su 9 was immunoprecipitated and the precipitates were analyzed by electrophoresis. Bands were visualized by fluorography and quantified by densitometry of exposed films. Processing peptidase activity was determined by calculating the sum of intermediate and mature forms of Su 9. Marker enzymes were assayed as described in Materials and Methods. For each digitonin concentration, the activities recovered from mitochondrial pellets and supernatants were set to 100%. The percentage of enzyme activity released from mitochondria is plotted versus the amount of digitonin used. (×——×) Adenylate kinase (Adk); (○– – –○) fumarase; (●——●) processing peptidase (Proc. Pept.); (×——×) succinate-cytochrome-*c*-reductase (Succ-Cyt c-Red)

Processing peptidase is a matrix-located activity

In order to determine the submitochondrial location of processing peptidase and especially to answer the question, whether both processing steps occur in the same subcompartment of mitochondria, we developed a fractionation procedure for small amounts of *Neurospora* mitochondria. Based on a protocol published previously [28], we used increasing concentrations of digitonin to differentially solubilize outer and inner membrane under conditions that preserved processing peptidase activity. This was only possible by employing a cytosolic protease inhibitor fraction from *Neurospora* (see Materials and Methods). The procedure allowed us to separate intermembrane space, matrix and inner membrane from each other as judged by the separation of marker enzyme activities. These enzymes were adenylate kinase, fumarase, and succinate-cytochrome *c*-reductase, respectively. In Fig. 4, the release of enzyme activity from the mitochondria is plotted versus the amount of digitonin used. Processing peptidase activity was estimated by calculating the sum of processing products [$m + i$, see above) after densitometry of exposed films. The band pattern obtained with the fraction containing the highest activity was essentially the same as that of Fig. 1 A, lane 5. Processing peptidase fractionated with the matrix marker fumarase.

To exclude the possibility that processing peptidase is associated with the outer membrane whose distribution was not followed in the experiment of Fig. 4, mitochondria were fractionated into membranes and soluble material in the pres-

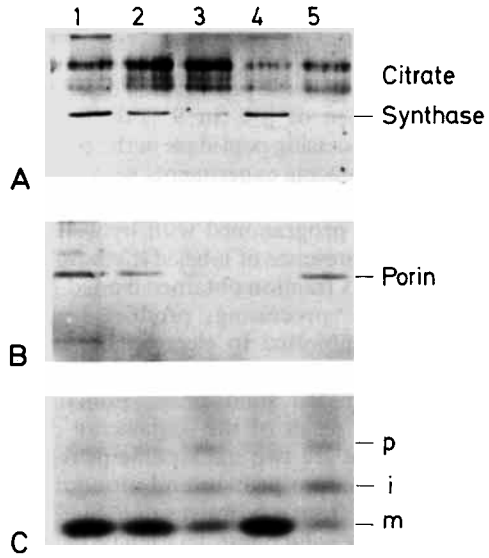

Fig. 5. *Processing peptidase is water-soluble.* Mitochondria were sonicated and centrifuged to separate membranes from the water-soluble fraction. The distribution of the matrix marker citrate synthase (A) and the outer membrane marker porin (B) was monitored by immunoreplica analysis. Lanes 1–3, 10, 5, 2.5 μg, respectively, of untreated mitochondria; lane 4, the supernatant fraction, corresponding to 10 μg of untreated mitochondria; lane 5, membrane fraction, corresponding to 10 μg of untreated mitochondria. (C) The fractions were assayed for processing peptidase activity in aliquots of desalted lysate containing radiolabeled precursors (100 μl each) in a final volume of 225 μl for 1 h. Su 9 was immunoprecipitated and subjected to electrophoresis. Bands were visualized by fluorography. Lanes 1–3, 25, 12.5, 6.25 μg, respectively, of untreated mitochondria; lane 4, supernatant fraction, corresponding to 25 μg of untreated mitochondria; lane 5, membrane fraction, corresponding to 26 μg of untreated mitochondria. p, precursor; i, intermediate; m, mature Su 9

ence of cytosolic protease inhibitor fraction (Fig. 5). The distribution of the matrix marker citrate synthase and the outer membrane marker porin [17] was monitored by Western blotting. Again, processing peptidase fractionated with the matrix marker.

The coincidence of the matrix marker enzymes and the processing activity strongly suggests that processing peptidase is a water-soluble activity located in the mitochondrial matrix.

Processing peptidase acts differently on various precursor proteins

The processing of precursor proteins others than pre Su 9 was investigated in the standard processing asssay. Radiolabeled reticulocyte lysates were incubated with mitochondrial extract for 1 h. The samples were immunoprecipitated for F_1-ATPase subunit β ($F_1\beta$), cytochrome c_1, and the Fe-S protein of the bc_1-complex (ubiquinol-cytochrome *c*-reductase) (Fig. 6). The precursor to $F_1\beta$ was processed to the mature-sized polypeptide. The precursor to cytochrome c_1 was processed to an intermediate-sized band. This intermediate was observed in intact cells and in whole mitochondria *in vitro* [5]. Further processing has been shown to require the covalent addition of a heme group and the action of a protease different from the metal-dependent matrix protease [6]. The precursor to the Fe-S protein was not processed by processing peptidase extracted from mitochondria. The reason for this is not known. One possibility is that this precursor is easily denatured under the conditions used, so that is no longer a substrate for processing peptidase. It is equally possible that a prerequisite for the proteolytic processing is the addition of Fe which might not work in our system, or that a specific membrane precursor orientation is required which is not available in our assay system.

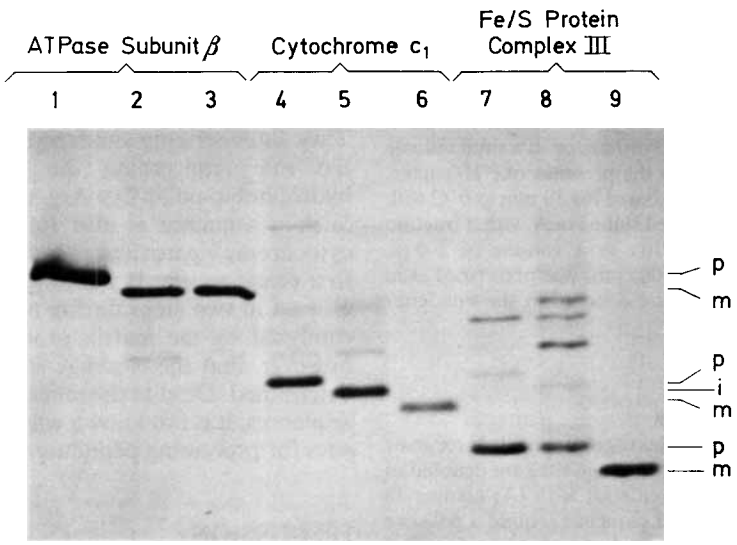

Fig. 6. *Processing of various mitochondrial precursor proteins.* Aliquots of desalted lysate containing radiolabeled precursors (100 μl each) were incubated with 20 μl of mitochondrial extract for 1 h in a final volume of 225 μl. The samples were immunoprecipitated for ATPase subunit β, cytochrome c_1 and the Fe/S protein. The precursors were immunoprecipitated from untreated lysates and the respective mature proteins from mitochondria labeled *in vivo*. After electrophoresis, the bands were visualized by fluorography. Lanes 1–3, ATPase subunit β, lanes 4–6, cytochrome c_1; lanes 7–9, Fe/S protein; lanes 1, 4, 7, precursors; lanes 2, 5, 8 processing products; lanes 3, 6, 9, mature proteins; p, precursor; i. intermediate; m, mature

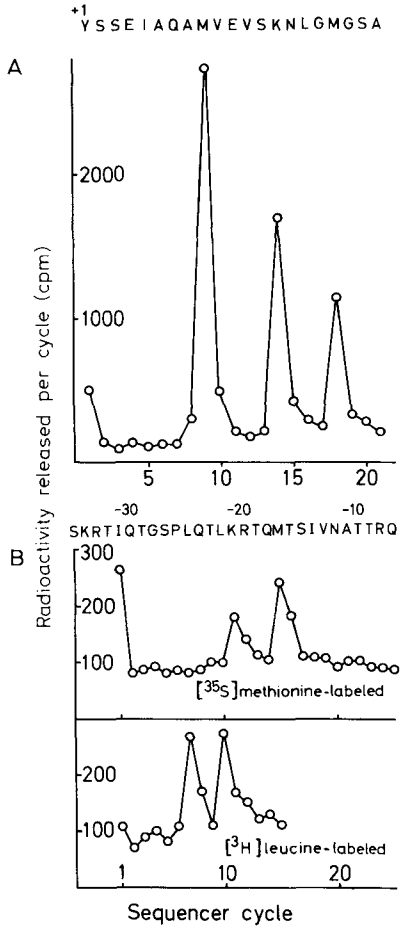

Fig. 7. *Sequence analysis of intermediate and mature-sized subunit 9 processed* in vitro. (A) The precursor to Su 9 was synthesized in a final volume of 20 µl of reticulocyte lysate programmed with hybridization selected mRNA in the presence of [^{35}S]methionine. The postribosomal supernatant was incubated for 1 h with a soluble fraction derived from 525 µg of digitonin-treated mitochondria under the same conditions as described in Materials and Methods. The final volume was 250 µl. Su 9 was immunoprecipitated and subjected to electrophoresis. Bands were visualized by fluorography. Mature-sized Su 9 was eluted from the gel (32400 cpm) and analyzed by solid-phase Edman degradation (B). Upper panel: intermediate sized Su 9 was eluted from the same gel as in A (12000 cmp) and analyzed as in A. Lower panel: the precursor to Su 9 was synthesized in a final volume of 50 µl of reticulocyte lysate as in A in the presence of [^{3}H]leucine. The postribosomal supernatant was incubated for 30 min at 0 °C with antibodies to Su 9. Processing was carried out as in A with a fraction corresponding to 360 µg of mitochondria in a volume of 200 µl. Analysis of intermediate sized Su 9 (15000 cpm) was performed as in A. Amino acid sequences of pre Su 9 are denoted in the one letter code

Table 1. *Comparison of the cleavage sites*
The amino acid sequences around the cleavage sites in the precursor to Su 9 (A, first cleavage site; B, second cleavage site) are denoted in the three letter code. The sequences given start with (A) alanine-38 and (B) glutamine-7. (C) The amino acid sequence around a putative cleavage site in the precursor to yeast cytochrome *c* peroxidase [29] starting with arginine-56 is presented. The cleavage sites are indicated by an arrow

A	Ala-Gln-Val-Ser-Lys-Arg-Thr-Ile -Gln-Thr-Gly
B	Gln-Ala-Phe-Gln-Lys-Arg-Ala-Thr-Ser -Ser -Glu
C	Arg-Thr-Ala-His-Lys-Arg-Ser -Leu-Tyr-Leu-Phe

Processing peptidase cleaves the precursor to ATPase subunit 9 at the correct site

Molecular cloning made possible the determination of the amino acid sequence of pre Su 9 [14]. To determine the cleavage sites of processing peptidase in the pre Su 9 molecule, we carried out sequencing experiments with mature-sized and intermediate-sized Su 9. The precursor was synthesized in reticulocyte lysates programmed with hybridization-selected mRNA [14] in the presence of labeled methionine. After processing with a matrix fraction obtained from digitonin-treated mitochondria, the processing products were immunoprecipitated, and subjected to electrophoresis. Mature and intermediate forms of Su 9 were eluted from the gel and subjected to automated solid phase Edman degradation. Fig. 7A shows the result of the sequencing of the mature-sized protein. There are two methionine peaks at position 9 and 18 as in the mature protein and an additional peak at position 14 coming from a lysine residue. Su 9 is coupled via lysine residues to the solid phase, so each lysine must also give a signal [25]. This result clearly indicates that the correct amino terminus is produced by processing peptidase. When the intermediate was sequenced, one lysine and one methionine peak was observed in the 11th and 15th cycle, respectively. In this experiment, extensive washing of the solid phase after binding of intermediate Su 9 was omitted. Thus, unspecifically absorbed material was eluted during the first Edman step resulting in a peak that does not tail. There is only one possibility that this pattern fits into the sequence of pre Su 9: the NH_2-terminus of the intermediate is isoleucine in position −31 (Fig. 7B, upper part). To confirm this interpretation, leucine-labeled intermediate was sequenced (Fig. 7B, lower part). This intermediate was obtained by carrying out the processing assay with precursor that had been incubated with antibodies to Su 9. Under these conditions the precursor was quantitatively processed only to the intermediate, and no mature Su 9 was observed (data not shown). Two leucine peaks were detected in the 7th and 10th cycle. The sequence -Leu-Xaa-Xaa-Leu- occurs only once in the whole precursor molecule between the positions leucine-25 and leucine-21. This again strongly suggests that isoleucine-31 is the NH_2-terminal amino acid of the intermediate.

Table 1 summarizes these results. The amino acid sequences around the cleavage sites of pre Su 9 are presented. They show striking similarities in that they appear to follow the rule (concerning the properties of amino acids): hydrophobic-polar-Lys-Arg-small- - - -bulky hydrophobic. Such a sequence is also found in the precursor to yeast cytochrome *c* peroxidase [29] that seems to indicate a putative first cleavage site. It was suggested [6] that this precursor is cleaved in two steps during its assembly, the first step being catalyzed by the matrix protease. It should be mentioned, however, that the cleavage site in this protein has not been determined. Despite the remarkable homology between these sequences, it is not known whether they represent recognition sites for processing peptidase.

DISCUSSION

The processing peptidase present in extracts of mitochondria cleaves the precursor to ATPase subunit 9 in a specific manner, so that the correct NH_2-terminus of the mature protein is produced. Processing peptidase is a metal-dependent enzyme located in the mitochondrial matrix. Proteolytic processing activities from different sources have been

described [7–10, 30, 31]. These activities show a high degree of homology in function and properties when compared to *Neurospora* processing peptidase. Processing peptidase appears to be responsible for the cleavage of a number of precursor proteins that are destined for different submitochondrial compartments. It is not known, however, how many different peptidases in the matrix of mitochondria are required for the processing of the probably several hundred different precursor proteins. Interestingly, yeast mitochondria contain a processing activity that cleaves *Neurospora* pre Su 9 correctly [15], although the homologous protein in yeast is made inside the mitochondria [32]. This implies that there may be only a limited number of processing peptidases or even only one such enzyme. This would be similar to the situation with secreted proteins in the endoplasmic reticulum and in bacteria [33, 34].

The matrix location of processing peptidase implies that pre Su 9 has to cross the inner mitochondrial membrane partially during its assembly to expose the NH_2-terminal extra sequence to the matrix. The rest of the precursor molecule appears to be firmly bound to the inner membrane [16]. There is some evidence that the NH_2-terminus of mature Su 9 faces the intermembrane space [35]. This would mean that the NH_2-terminus has to be translocated back across the inner membrane after the second processing.

The mitochondria-free system used in this work produced a kinetic intermediate during processing of pre Su 9. A two-step processing of pre Su 9 was expected since precursor transfer experiments *in vitro* with intact mitochondria have revealed an intermediate when processing was slowed down in the presence of chelating agents [16]. In the absence of such chelators the intermediate has not been observed [11]. Probably under normal conditions the rate-limiting step is the first cleavage. The *in vivo* processing experiments presented in this paper indicate that intermediate sized Su 9 is not an *in vitro* artifact, but represents a true intermediate in the assembly pathway of Su 9. No additional processing steps between precursor and intermediate and between intermediate and mature Su 9 have been observed. However, additional cleavages cannot be ruled out *a priori*. Indeed, there is a sequence between intermediate and mature forms which is rather similar to the two established cleavage sites (around threonine-19 and glutamine-18). The physiological meaning for the two-step processing is not known. It is remarkable, however, that the additional sequence of pre Su 9 is exceptionally large.

The two cleavage sites in the pre Su 9 molecule are homologous to each other as judged from the similarities in primary structure. This type of structure is also found in the precursor to yeast cytochrome *c* peroxidase (see Table 1). This sequence may be the site of cleavage leading to the formation of an intermediate in cytochrome *c* peroxidase assembly. There are, however, no structural data available to substantiate this proposal. As only a few sequences of mitochondrial precursor proteins are available, it is difficult to tell what characteritic of this type of sequence is the minimal requirement for the recognition of these sites by mitochondrial processing peptidase. The precursor of yeast mitochondrial EF-Tu [36] does not contain such a sequence and the question remains to be answered, whether this precursor is processed by the same enzyme that cleaves the precursor to yeast cytochrome *c* peroxidase.

There are several lines of evidence that pre Su 9 is cleaved in two steps by the same enzyme: (a) both processing steps take place in the same subcompartment of mitochondria, i.e. the matrix; (b) both steps show the same sensitivity to inhibitors; (c) the cleavage sites are very similar to each other. Other precursor proteins that are cleaved in two steps appear to require two different proteolytic processing enzymes from probably different submitochondrial locations. This has been shown for cytochrome c_1 [5, 6] in *Neurospora* and yeast and for cytochrome b_2 [6, 10] in yeast. It is also probably true for yeast cytochrome *c* peroxidase [6]. The submitochondrial location of the second processing enzyme is not clear, because an assay in a mitochondria-free system is not yet available.

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