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Import of the Iron-Sulfur Protein of the Cytochrome b-\textit{c}_1 Complex into Yeast Mitochondria*

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The yeast gene for the Rieske iron-sulfur protein of the cytochrome b-\textit{c}_1 complex was subcloned into the expression vector, pSP64, then transcribed and translated \textit{in vitro} in a reticulocyte lysate in the presence of \textsuperscript{35}S)methionine. Import studies \textit{in vitro} of the newly synthesized precursor form of the iron-sulfur protein into isolated yeast mitochondria revealed that the precursor form of the iron-sulfur protein is processed into the mature form via an intermediate form. After the import reaction at 18 or 27 °C, treatment of mitochondria with exogenous protease indicated that both intermediate and mature forms had been internalized into mitochondria where they were resistant to digestion by external protease. Import and processing of the iron-sulfur protein into mitochondria also occurred at temperatures ranging from 2 to 27 °C in a temperature-dependent manner. Processing of the precursor form to the intermediate form appeared to be less sensitive to temperature than the processing of the intermediate form to the mature form. Moreover, at temperatures of 12 °C or lower, the mature form produced was completely digested by exogenous protease suggesting that it was assembled incorrectly in the membrane and not assembled into the b-\textit{c}_1 complex. The successive disappearance of first the mature form and then the intermediate form of the iron-sulfur protein by increasing concentrations of the metal chelators, EDTA and o-phenanthroline, suggested that two different proteases requiring divalent metal ions are involved in the two-step processing of the precursor form of the iron-sulfur protein. Furthermore, mitoplasts containing only the matrix/inner membrane fraction were able to import and process the precursor form of the iron-sulfur protein indicating that both proteolytic processing events occur in the matrix/inner membrane fraction.

The majority of mitochondrial proteins are encoded by nuclear genes and synthesized on free cytoplasmic ribosomes, despite the fact that mitochondria contain their own unique DNA. Mechanisms of import and processing of nuclear-coded mitochondrial proteins have been extensively investigated since the discovery in 1979 (1) that subunits of the F\textsubscript{1}-ATPase are synthesized in the cytosol as larger precursor proteins containing amino-terminal extensions (presequences) prior to import into mitochondria. Subsequent studies have provided evidence that the import and processing of precursor proteins into mitochondria occur through several distinct steps (2-6). After completion of translation, precursor forms of mitochondrial proteins containing targeting signals (amphiphilic structures composed of hydrophobic regions linked by basic residues) are directed to the mitochondrial outer membrane (6-9). A second step in the import pathway requires the binding of these precursor proteins to proteinaceous receptors exposed at the cytoplasmic face of the outer membrane (10-12). The precursor proteins are then unfolded by a mechanism involving ATP to a translocation-competent conformation (13, 14). The eventual translocation of precursor proteins into the mitochondrial matrix via contact sites between the outer and inner membranes is dependent on an electrochemical potential (\(\Delta \psi\)) across the inner membrane (15). During or after translocation, precursor proteins are processed to their mature forms by either one or two separate cleavages by matrix-localized protease(s) (16-18). Finally, the processed mature proteins are assembled into functional complexes in their proper subconidochondrial compartment.

The Rieske iron-sulfur protein, one of the catalytic subunits of the cytochrome b-\textit{c}_1 complex, is localized on the outer surface of the inner mitochondrial membrane (19, 20). This protein contains two Fe-S clusters and is required for electron transfer from ubiquinol to cytochrome \textit{c}_1 in the b-\textit{c}_1 complex (21). Our laboratory made the initial observation in intact yeast cells that the precursor form of the iron-sulfur protein is processed \textit{in vivo} into the mature form via an intermediate form (22). This proteolytic two-step cleavage of the precursor iron-sulfur protein was subsequently observed during experiments both \textit{in vitro} and \textit{in vivo} in \textit{N. crassa} (23). Moreover, the intermediate form of the iron-sulfur protein was shown to be localized in the matrix fraction as it fractionated with the matrix marker fumarase upon treatment of mitochondria with digitonin (23).

Recently, the nuclear gene encoding the Rieske iron-sulfur protein has been isolated and sequenced from both \textit{N. crassa} (24) and \textit{S. cerevisiae} (25). In the current study, we have subcloned the iron-sulfur protein gene into a pSP64 expression vector to study the import and processing of the precursor form of the iron-sulfur protein \textit{in vitro} into isolated yeast mitochondria. The results obtained confirm the two-step cleavage of the precursor iron-sulfur protein in yeast mitochondria and suggest different temperature sensitivities for each step. Moreover, treatment of mitochondria with exogenous protease after import has indicated that both intermediate and mature forms of the iron-sulfur protein are localized in a protease-resistant compartment. The precursor form of the iron-sulfur protein can also be imported and processed to both intermediate and mature forms in mitoplasts (mitochondria from which the outer membrane has been removed) indicating that both processing steps occur in the inner membrane/matrix fraction.
Iron-sulfur protein precursor was centrifuged at 105,000 x g for 45 min and incubated with isolated yeast mitochondria at 23°C (A) or 18°C (B) for the times as indicated. Mitochondria were added at a final concentration of 1.0 mg/ml for each import reaction. A, at each time point, 300 μl of import mixture was withdrawn and analyzed as described under "Experimental Procedures." B, one-half of each import mixture (400 μl) was removed (lanes 1–6) as described in A while the remaining half was treated with proteinase K (lanes 7–12). The positions of precursor (p), intermediate (i), and mature (m) forms of the iron-sulfur protein are indicated. C, the bands of the three forms of the iron-sulfur protein from the autoradiograph of the gel shown in B, lanes 1–6, were quantified by laser densitometry. The mature form observed at 30 min was arbitrarily set at 100.

EXPERIMENTAL PROCEDURES AND RESULTS

Import of the Iron-Sulfur Protein Precursor—The time course of import of the precursor form of the iron-sulfur protein into mitochondria was studied by incubating yeast mitochondria with translation mixtures containing radiolabeled precursor. At the times indicated, aliquots of the import mixture were withdrawn and analyzed by SDS-PAGE with or without proteinase K treatment. Fig. 4A shows that the import and processing of the iron-sulfur protein into mitochondria occurred rapidly at 27°C. Maximum amounts of the labeled mature form were observed after a short incubation, times of 1 or 2 min, while only traces of labeled intermediate form were observed at all times studied. When the import incubation was performed at 18°C, the presence of the intermediate form became apparent. Labeling of both the intermediate and mature forms increased with time in a parallel fashion with the mature form more heavily labeled at each time point. The amount of the precursor form remained essentially constant for 20–30 min reflecting continued binding of the precursor form to the mitochondria during this time as processing occurred (Fig. 4B, lanes 1–6). Consequently, the total amount of radiolabeled iron-sulfur protein, the sum of the precursor, intermediate, and mature forms, increased more than 50% from the 2- to the 20-min time point at which time a decrease in the amount of precursor was observed. At all times of import at 18°C, the precursor form was digested by exogenous proteinase K; however, both the intermediate and mature forms were resistant to digestion by exogenous proteinase K indicating that they are present in a protease-resistant compartment of the mitochondria (Fig. 4B, lanes 7–12). A slight loss in the amount of the mature form after proteinase K digestion suggests possible damage to the outer membrane of the mitochondria allowing some digestion of the mature form which is exposed to the outer surface of the inner membrane.

The profound effect of temperature on the import and processing of the iron-sulfur protein into yeast mitochondria prompted us to examine the effect of even lower temperatures on the import reaction. Clearly, import and processing of the iron-sulfur protein into yeast mitochondria were temperature-dependent (Fig. 5, compare lanes 1, 4, and 7). Both the intermediate and mature forms of the iron-sulfur protein were resistant to proteinase K treatment after import at 27°C (lane 8), suggesting that both forms have been internalized at this temperature. Surprisingly, mature form as well as the precursor form were accessible to exogenous protease when the import reaction was performed at 2 or 12°C (lanes 2 and 5); however, the intermediate form remained resistant to protease treatment after the import at these temperatures. The intermediate form produced at the lower temperatures of incubation appears more diffuse after proteinase K digestion, but does not represent digested precursor form as it is clearly localized in the matrix as discussed under "Submitochondrial Matrix."
Localization of the Different Forms of the Iron-Sulfur Protein. Moreover, the amount of intermediate remains the same before and after proteinase K digestion which would be unlikely if the intermediate form represented digested precursor form. After the initial import of the precursor form at 2 or 12 °C, the incubation mixture was further incubated for 20 min at 27 °C in the presence of antimycin A and oligomycin. The chase at 27 °C resulted in the processing of the precursor and intermediate forms to the mature form which was then resistant to added proteinase K (lanes 3 and 6). These data suggest that the processing of the intermediate to the mature form is more sensitive to temperature than the processing and import of the precursor form into a protease-resistant intermediate form.

The sensitivity of the intermediate and mature forms of the iron-sulfur protein to exogenous proteinase K was further explored by studying the time course of import at 12 °C. The results obtained confirm that only the intermediate form remained resistant to exogenous proteinase K after import at 12 °C (Fig. 6, A and B), while at all times of incubation the mature form was sensitive to digestion. As mentioned above, the intermediate form labeled under these conditions appeared very diffuse after proteinase K digestion for reasons which remain obscure. Perhaps the protease has attached a portion of the intermediate form such that it now runs more slowly on the gel. However, the absolute amounts of the intermediate form before and after proteinase K digestion are almost identical at all time points examined indicating that no loss of intermediate form resulted from treatment with the exogenous protease. The appearance of both intermediate and mature forms increased with time during the incubation reaching a maximum after 40 min. The amount of the precursor form remained constant throughout the incubation 20-min incubation was normalized to 100 units.

In Fig. 4 was presented the time course of import of the iron-sulfur protein precursor into mitochondria during the incubation at 12 °C; however, the processed mature form produced at 12 °C is assembled incorrectly in the mitochondrial membrane where it remains accessible to proteinase K.

Effects of Inhibitors on Import of the Iron-Sulfur Protein—The effects of inhibitors which block formation of a membrane potential plus chelators shown previously to inhibit the matrix-localized mitochondrial processing proteases were studied during import of the iron-sulfur protein precursor into mitochondria at 18 °C. In the presence of antimycin A and oligomycin, only the precursor form was observed in the reisolated mitochondria. In addition, the precursor was completely digested by proteinase K treatment (Fig. 7A, lanes 5 and 10) indicating that the import and processing of the iron-sulfur protein requires the presence of a membrane potential. Addition of 10 mM EDTA, a nonpenetrating metal chelator, or 0.5 mM o-phenanthroline, a membrane-permeable metal chelator, to the import mixture separately had little effect on processing of the precursor form (lanes 2, 3 and 7, 8); however, addition of both EDTA and o-phenanthroline caused a considerable inhibition of the processing of the intermediate to the mature form (lanes 4 and 9). Some inhibition of processing the precursor to the intermediate form was also observed, but the precursor form remained protease-sensitive (lane 9).

Increasing concentrations of both EDTA and o-phenanthroline added to the import reaction at 18 °C resulted in the successive disappearance of first the mature and then the intermediate form of the iron-sulfur protein (Fig. 7B). The addition of 2.5 mM EDTA and 0.5 mM o-phenanthroline (lane 1) inhibited partially the conversion of intermediate to mature form. Increasing the concentration of EDTA to 10 mM and o-phenanthroline to 2 mM completely inhibited the conversion of intermediate to mature form and partially inhibited the conversion of precursor to intermediate form (lane 3). Furthermore, when the concentration of o-phenanthroline was increased to 4 mM, processing of the precursor to intermediate form was inhibited completely.
form was almost completely blocked. As the processing protease converting the precursor to the intermediate form was inhibited, the precursor form became resistant to exogenous protease suggesting that it had been translocated into the mitochondria (data not shown).

**Submitochondrial Localization of the Different Forms of the Iron-Sulfur Protein**—To localize both the intermediate and mature forms of the iron-sulfur protein within the mitochondria, mitochondria were subfractionated by digitonin treatment after the import reaction. Enzymes present in the intermembrane and the matrix spaces are released sequentially by successive increases in digitonin concentration. Fumarase was used as an enzymatic marker for the mitochondrial matrix. Mature form was almost completely blocked. As the processing protease converting the precursor to the intermediate form was released after treatment with 0.15% digitonin, which released 75% of the intermediate form and all of the mature form. These results suggest that the intermediate form of the iron-sulfur protein is present in the matrix space. The mature form, however, is more susceptible to solubilization by higher concentrations of digitonin suggesting that it is not in the matrix but loosely associated with the inner membrane. Interestingly, an identical release of the intermediate and mature forms with increasing concentrations of digitonin was observed after import at 12 and 27 °C, as well as at 18 °C (Fig. 8).

**Import of the Iron-Sulfur Protein into Mitoplasts**—Import and processing of the precursor form of the iron-sulfur protein into isolated mitochondria had been successfully demonstrated at temperatures ranging from 4 to 27 °C. We next attempted to demonstrate whether the precursor form of the iron-sulfur protein could be imported into mitoplasts which lack the mitochondrial outer membrane. The time course of the import of the precursor form of the iron-sulfur protein into mitoplasts at 18 °C indicated that mitoplasts can import the precursor form as efficiently as mitochondria (Fig. 9). Both the intermediate and the mature forms were observed in the absence of proteinase K during the different time intervals suggesting that both proteases involved in the conversion of the precursor form to the intermediate form and the intermediate form to the mature form are present in the mitochondrial matrix-inner membrane fraction. The intermediate form was resistant to digestion by exogenous proteinase K, while both precursor and mature forms were sensitive to digestion suggesting that conversion of the intermediate form into the mature form occurred in the matrix/inner membrane fraction. Moreover, the mature form is assembled on the outer surface of the inner mitochondrial membrane where it was accessible to exogenous proteinase K.

Next, the inhibitors of the proteases involved in the two-step cleavage of the iron-sulfur protein were studied during the import reaction in mitoplasts (Fig. 10). Raising the concentrations of EDTA and o-phenanthroline resulted in the successive inhibition of the two proteases as previously observed in mitochondria (Fig. 7B). After addition of exogenous proteinase K (lanes 6–9), the intermediate form remained resistant to proteinase K. As the processing enzyme converting precursor to intermediate form became inhibited, the precursor form also became resistant to proteinase K. The import of the precursor iron-sulfur protein into mitoplasts was investigated at 4, 12, and 18 °C (Fig. 11, lanes 1, 2, and 3, respectively). Only the precursor form of the iron-sulfur protein was observed at 4 and 12 °C in contrast to the results obtained using mitochondria (Fig. 5). Significant import and processing to both intermediate and mature forms were ob-

![Fig. 8](image-url) **Fig. 8.** Release of both imported intermediate and mature forms of the iron-sulfur protein from mitochondria by treatment with digitonin. Mitochondria were incubated with the newly synthesized iron-sulfur protein at 18 °C for 20 min. The reaction mixture was divided into 8 equal portions and mitochondria were reisolated by centrifugation. The reisolated mitochondria were washed twice with a buffer containing 0.6 M sorbitol and 20 mM Hepes/KOH (pH 7.4) and then resuspended at 0.2 ml of above buffer in the presence of the indicated concentrations of digitonin as indicated under “Experimental Procedures.” Each fraction was separated into pellet and supernatant by centrifugation. The pellets were analyzed by SDS-PAGE while the supernatants were assayed for fumarase activity. An autoradiograph of the dried gel is shown in A. The bands of both intermediate and mature forms of the iron-sulfur protein quantified by laser densitometry and the fumarase activities are shown in B. Abbreviations are as in Fig. 4.

![Fig. 9](image-url) **Fig. 9.** Time course of import of the iron-sulfur protein into mitoplasts. Translation mixture containing 35S-labeled precursor iron-sulfur protein was incubated with mitoplasts prepared as described under “Experimental Procedures” at 18 °C for the indicated times. Abbreviations are as in Fig. 4.
in vitro served at 18 °C. In addition, when the membrane potential was blocked by the addition of antimycin A and oligomycin as into mitochondria. This experiment was identical with that of Fig. 7B, except that mitoplasts were used in the incubation. 

![Fig. 10](image)

**Fig. 10.** Effect of EDTA and o-phenanthroline on the proteolytic two-step processing of the iron-sulfur protein in mitoplasts. This experiment was identical with that of Fig. 7B, except that mitoplasts were used in the incubation. Abbreviations are as in Fig. 4.

![Fig. 11](image)

**Fig. 11.** Effect of different temperatures on the import of the iron-sulfur protein into mitoplasts. The import reactions were performed at various temperatures: 4 °C (lane 1), 12 °C (lane 2), and 18 °C (lanes 3–5). Antimycin A and oligomycin were added to the import mixture in lane 4, while 10 mM EDTA and 2.0 mM orthophenanthroline were added to lane 5. After the import incubation, the reisolated mitoplasts were analyzed by SDS-PAGE. Abbreviations are as in Fig. 4.

served at 18 °C. In addition, when the membrane potential was blocked by the addition of antimycin A and oligomycin (lane 4), the import of the precursor form into mitoplasts was inhibited suggesting that a membrane potential is necessary for the import of the precursor form into mitoplasts as well as into mitochondria.

**DISCUSSION**

Both intermediate and mature forms of the iron-sulfur protein were observed during import studies in vitro of the newly synthesized precursor form of this protein into isolated yeast mitochondria. These results confirm previous reports obtained in yeast in vivo (22) and N. crassa both in vivo and in vitro (23) indicating that the precursor form of the iron-sulfur protein is processed to the mature form via an intermediate form. This apparent two-step proteolytic processing has also been reported for other mitochondrial proteins encoded by nuclear genes and synthesized in the cytoplasm such as cytochrome c (33,34), cytochrome b (34,35), and subunit 9 of ATPase (36). Treatment of mitochondria after the import reaction with exogenous protease or digitonin extraction suggested that both intermediate and mature forms have been internalized into mitochondria at 18 or 27 °C where they are insensitive to protease digestion and released by digitonin treatment concomitantly with the matrix marker fumarase. Moreover, the precursor form of the iron-sulfur protein was imported and processed to both intermediate and mature forms in mitoplasts indicating that both proteases are active in mitoplasts. Further lowering of the temperature of the inner membrane is incorrectly translocated by the 18 °C temperature to permit observation of the intermediate form. At import temperatures at or below the transition temperature of the inner membrane is incorrectly translocated back across the membranes perhaps via the contact sites, where it becomes accessible to protease K. Previous studies in N. crassa had indicated that at 2 °C to 8 °C no formation of the mature form of the iron-sulfur protein occurred (23).
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In that system, all species of the iron-sulfur protein were largely sensitive to added proteinase K at incubation temperatures below 12 °C (23).

The results of this study suggest that the precursor form of the iron-sulfur protein in yeast first associates tightly with the mitochondrial membrane and is translocated into the matrix in a process dependent on an electrical potential across the inner membrane. In the matrix space, the precursor form is first processed into the intermediate form by a matrix protease. The subsequent processing of the intermediate form to the mature form is catalyzed by a second protease located in the matrix/inner membrane fraction and appears to be coupled to the translocation of the mature form back across the inner membrane where it is assembled into the b-c1 complex. The exact intramitochondrial localization of the second protease and the mechanism of assembly of the mature form of the iron-sulfur protein with other subunits of the b-c1 complex is currently under investigation in our laboratory.

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The pellets were analyzed by SDS polyacrylamide gel electrophoresis and fluorography where the supernatants were assayed for luminescence activity (30).

**Molecular Methods**

Plasmid construction, agarose gel electrophoresis were performed by standard techniques as described by Maniatis et al. (24). Autoradiograms were quantified by measuring relative optical densities using a Bioimage laser scanning densitometer.

**Materials**

L-[35S]methionine (1000–1400 Ci/mmol) was obtained from Amersham Corp. Nuclear-purified ribosome reticulocyte lysate and amino acid mixture minus methionine were purchased from Promega. HEPES, EDTA, anti-myc antibody, methionine-free, TCOY, glycine, and glutamine were from Sigma.  

plasmid was from Invitrogen and Roche, respectively.

**RESULTS**

Transcription and translation of pSP64-RIP

The identity of the recombinant plasmid pSP64-RIP was confirmed by restriction analysis using the endonucleases SacI, HindIII and PstI in various combinations. Figure 2 indicates that the plasmid was digested with HindIII and EcoRI, as predicted (lane 2). Digestion of the plasmid with both SacI and HindIII resulted in the ligation of a 2 and a 3 kb fragment (lane 3). In addition, fragments of 4.48 and 6.32 kb (lane 4) and 3.52 and 1.48 kb (lane 5) were observed, confirming the digestion of pSP64-RIP with HindIII and SacI, respectively. These results indicate that the RBP gene has been successfully subcloned into the expression vector pSP64.

![Figure 2](image-url)  

**Fig. 2.** Restriction analysis of the plasmid. The pSP64-RIP was digested with HindIII, SacI, and PstI in various combinations. The fragment sizes reported are given in kb. **A**. The SacI-digested pSP64-RIP plasmid was digested with HindIII and EcoRI in various combinations. The fragment sizes reported are given in kb. **B**. The HindIII-digested pSP64-RIP plasmid was digested with SacI and PstI in various combinations. The fragment sizes reported are given in kb. **C**. The EcoRI-digested pSP64-RIP plasmid was digested with SacI and PstI in various combinations. The fragment sizes reported are given in kb.

The linearized pSP64-RIP produced by the digestion of SacI was transcribed in vivo in the presence of [35S]methionine. The capped mRNA thus produced, was gel purified by electrophoresis and Northern blot hybridization. A single band corresponding to approximately 1.2 kb was observed (Fig. 3A, lane 1). This mRNA preparation was translated in a rabbit reticulocyte lysate in the presence of [35S]methionine. Analysis of the translation products by SDS-PAGE followed by autoradiography revealed a single band corresponding to 23.2 kDa (Fig. 3A, lane 2). A band with the same migration was also observed after immunoprecipitation of the translation mixture with specific antibodies against the iron-sulfur protein (lane 1). The observed molecular weight of the newly-synthesized radiolabeled polypeptide is similar to that reported previously for the precursor form of the iron-sulfur protein in yeast (22-31).

![Figure 3](image-url)

**Fig. 3.** In vitro transcription and translation of pSP64-RIP gene. A. The SacI-digested linearized pSP64-RIP was transcribed in vivo as described in "Materials." Various amounts of the capped mRNA were directly loaded and separated on a 1% agarose gel (lane 4) or transcribed onto nylon membrane (lanes 1–3). The Northern blots were hybridized with a nick translated DNA probe containing the PstI-EcoRI fragment of the RBP gene. B. Two of the translation mixtures were used for immunoprecipitation with an anti-myc antibody, as described in "Materials." Two of the translation mixtures were used for immunoprecipitation with an anti-myc antibody, as described in "Materials." Two of the translation mixtures were used for immunoprecipitation with an anti-myc antibody, as described in "Materials." Two of the translation mixtures were used for immunoprecipitation with an anti-myc antibody, as described in "Materials."