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Assembly of the Iron-Sulfur Protein into the Cytochrome b-c_1, Complex of Yeast Mitochondria*

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The assembly of the iron-sulfur protein into the cytochrome bc_1 complex after import and processing of the precursor form into mitochondria *in vitro* was investigated by immunoprecipitation of the radiolabeled iron-sulfur protein from detergent-solubilized mitochondria with specific antisera. After import *in vitro*, the labeled mature form of the iron-sulfur protein was immunoprecipitated by antisera against both the iron-sulfur protein and the entire bc_1 complex from mitochondria solubilized with either Triton X-100 or dodecyl maltoside. After sodium dodecyl sulfate solubilization of mitochondria, however, the antiserum against the iron-sulfur protein, but *not* that against the bc_1 complex, immunoprecipitated the radiolabeled iron-sulfur protein. These results suggest that in mitochondria the mature form of the iron-sulfur protein is assembled with other subunits of the bc_1 complex that are recognized by the antiserum against the bc_1 complex. By contrast, the intermediate and precursor forms of the iron-sulfur protein that accumulated in the matrix when proteolytic processing was blocked with EDTA and *o*-phenanthroline were not efficiently assembled into the bc_1 complex. The import and processing of the iron-sulfur protein also occurred in mitochondria lacking either cytochrome *b* (W-267) or the iron-sulfur protein (JPJ1). The mature form of the iron-sulfur protein was immunoprecipitated by antisera against the bc_1 complex or core protein I after import *in vitro* into these mitochondria, suggesting that the mature form is associated with other subunits of the bc_1 complex in these strains.

The majority of mitochondrial proteins are encoded by nuclear genes, synthesized on free cytosolic ribosomes and, in a subsequent step, imported into mitochondria where they are processed and assembled into their functional units (for reviews see Refs. 1–6). Many mitochondrial proteins are synthesized as larger precursor forms containing amino-terminal extensions (presequences) that aid in directing these proteins to proteinaceous receptors present on the outer mitochondrial membrane (7–10). ATP and cytosolic cofactors, including a 70-kDa heat-shock protein (hsp70), are necessary to maintain these precursor proteins in an unfolded translocation-competent conformation (11–14). The precursor proteins bound to the mitochondrial membrane are then targeted to a common insertion site located in the contact sites formed between the outer and inner membranes. The protein components involved in membrane insertion at these contact sites have recently been identified in both *Neurospora crassa* and *Saccharomyces cerevisiae* (15, 16). The eventual translocation of these precursor proteins into the mitochondrial matrix requires a membrane potential across the inner membrane ($\Delta\psi$) plus another hsp70 located in the mitochondrial matrix (17, 18). During or after translocation, the precursor proteins are processed to their mature forms by one or two separate cleavages by matrix-localized protease(s) (19–21). The final step in the assembly of the imported proteins into functional complexes in the correct mitochondrial subcompartments involves a chaperonin-like protein, mitochondrial hsp60, which maintains these proteins in the proper conformation in an ATP-dependent process (22–24).

The mechanism of assembly of imported mitochondrial proteins into functional multi-enzyme complexes remains to be elucidated. Several years ago, we reported that the assembly of the different subunits of the cytochrome bc_1 complex into the holoenzyme occurred at very different rates (25, 26). More recently, it was suggested that the individual subunits are added to the cytochrome bc_1 complex in an ordered assembly process (27). An ordered and sequential assembly has also been suggested for the assembly of the cytochrome *c* oxidase complex of rat liver mitochondria (28) and of the F_1-ATPase of yeast mitochondria (29). Evidence for the assembly of individual subunits into the bc_1 complex and the F_1F_0-ATPase after import *in vitro* was obtained by using specific antisera against individual subunits. For example, an antiserum against cytochrome c_1 immunoprecipated labeled iron-sulfur protein from detergent-solubilized mitochondria (30), whereas an antiserum against F_1-ATPase immunoprecipitated subunit 9 of F_0 (31). The role of the different subunits of these complexes on the assembly of an intact complex has also been implicated in studies with individual subunits of the bc_1 complex. Deletions of cytochrome *b* and other subunits of the bc_1 complex resulted in the loss of several other subunits of the complex (27).

Our previous reports demonstrated that the precursor form of the iron-sulfur protein is processed in *S. cerevisiae* into the mature form via an intermediate form both *in vivo* and *in vitro* (26, 32). In the present study, the assembly of the iron-sulfur protein into the cytochrome bc_1 complex has been investigated by immunoprecipitation of the newly imported radiolabeled iron-sulfur protein from detergent-solubilized mitochondria with specific antisera against the iron-sulfur protein, core protein I, and the intact bc_1 complex. The results obtained indicate that the mature form of the iron-sulfur protein imported into isolated mitochondria *in vitro* has associated with other subunits of the bc_1 complex. By contrast, the intermediate and precursor forms of the iron-sulfur pro-

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tein that accumulate when processing is blocked by addition of the metal chelators EDTA and *o*-phenanthroline were not efficiently assembled into the bc_1 complex. In addition, we have also investigated the import and assembly of the iron-sulfur protein into a mutant yeast lacking cytochrome *b* and a strain of yeast in which the nuclear gene for the iron-sulfur protein has been disrupted (33).

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—The wild-type strain 777-3A, the cytochrome *b*-deficient strain W-267, and the iron-sulfur protein-deficient strain JPJ1 of *S. cerevisiae* were used in this study. The wild-type, 777-3A, and the mutant, W-267, yeast strains were grown aerobically at 30 °C in a semisynthetic medium in which lactate (22 ml/liter) and galactose (30 g/liter) were utilized as the carbon sources, respectively (34). The growth medium for the mutant strain JPJ1 contained yeast nitrogen base without amino acids (6.7 g/liter; Difco), adenine (80 mg/liter), uracil (20 mg/liter), histidine (20 mg/liter), tryptophan (20 mg/liter), and glucose (20 g/liter) as the carbon source (35). Cells were grown to the early logarithmic phase (OD_{650} = 0.9–1.2) and harvested by centrifugation. Mitochondria and mitoplasts were isolated as previously described (32).

Import of the Iron-Sulfur Protein into Mitochondria in Vitro—The import of radiolabeled iron-sulfur protein into mitochondria isolated from wild-type cells was assayed as described previously (32). For the import of labeled iron-sulfur protein into mitochondria of the mutant strains W-267 and JPJ1, the assay buffer contained 0.6 M sorbitol, 20 mM Hepes[1]/KOH (pH 7.4), 40 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM ATP, 8 mM ascorbate, 0.2 mM TMPD, 5 mM phosphoenolpyruvate, and 5 units of pyruvate kinase in a final volume of 0.2 ml. The incubation conditions for the mutant mitochondria were identical with that for wild-type mitochondria (32).

Sonication of Mitochondria—After the import incubation, mitochondria were reisolated by centrifugation at 10,800 × *g* for 5 min in a refrigerated microcentrifuge, suspended in 0.4 ml of a buffer containing 0.6 M sorbitol and 20 mM Hepes/KOH (pH 7.4), sonicated at 50% output three times for 5 s each in a Heat Systems-Ultrasonics Inc. model W-375 sonicator, and centrifuged at 100,000 × *g* for 30 min to obtain a pellet containing membranes.

Immunoprecipitation—After import of the labeled iron-sulfur protein into mitochondria *in vitro*, the washed mitochondria were solubilized with detergent using 3% SDS, 1% Triton X-100, 0.6 mg of dodecyl maltoside (DM)/mg of protein, or 0.5 mg of deoxycholate/mg of protein in 100 μl of a buffer containing 0.1 M Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The SDS-dissociated sample was vigorously shaken at room temperature for 30 min, whereas the samples treated with the other detergents were shaken at 4 °C for 30 min. Subsequently, all the samples were diluted 10-fold with the above buffer containing 1% Triton X-100 and then incubated with the indicated antiserum for 16 h at 4 °C. After centrifugation at 10,800 × *g* for 5 min, Sepharose-bound protein A (10–12 mg, dry weight, preswollen in 100 μl of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM EDTA) was added to the supernatants, and the mixture was incubated with shaking for 1 h at room temperature. The Sepharose beads were collected by centrifugation, then washed two times with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 0.2% Triton X-100 and two times with the same buffer without Triton X-100. The washed Sepharose-bound immunoprecipitates were resuspended in 100 μl of dissociation buffer and incubated for 5 min at 95 °C. The beads were removed by centrifugation at 10,800 × *g* for 3 min, and the supernatants were subjected to SDS-PAGE.

Miscellaneous Methods—Published procedures were used for the *in vitro* transcription and translation of pSP64-RIP (32). Specific antibodies against the cytochrome bc_1 complex, core protein I, and the iron-sulfur protein were prepared as previously described (25). SDS-PAGE was performed as described previously (32). Autoradiographs were quantitated by measuring relative optical densities using a Biolmage laser scanning densitometer.

Materials—L-[^{35}S]Methionine (1200 Ci/mmol) was obtained from ICN Biomedicals, Inc. Nuclease-treated rabbit reticulocyte lysate and amino acids mixture minus methionine were purchased from Promega Biotec. Protein A-Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. Prestained molecular weight markers were purchased from Diversified Biotech. HEPES, EDTA, phenylmethylsulfonyl fluoride, TMPD, proteinase K, phosphoenolpyruvate, and pyruvate kinase were from Sigma. *Ortho*-phenanthroline was obtained from Fisher. Deoxycholic acid and DM were obtained from Calbiochem.

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TMPD, tetramethylenephenyldiamine; DM, dodecyl maltoside.

RESULTS

Import and Assembly of the Iron-Sulfur Protein into the Cytochrome bc_1 Complex—Previously, we reported that in isolated yeast mitochondria the precursor form of the iron-sulfur protein is processed to the mature form via an intermediate form (32). Both the intermediate and mature forms are localized in a mitochondrial compartment where they are resistant to digestion by proteinase K; however, the precursor form is digested by the exogenous protease (Fig. 1, *lanes 1* and *2*). To learn whether the internalized forms of the iron-sulfur protein are assembled into the bc_1 complex, treatment of detergent-solubilized mitochondria with specific antisera was performed after import of radiolabeled precursor. Specific antisera raised against the iron-sulfur protein, core protein I and the intact bc_1 complex were used (36). Previous immunoblotting studies (35) had revealed that the latter antiserum recognized mainly core protein I, core protein II, cytochrome *b*, and cytochrome c_1 and, to a lesser extent, the smaller subunits of the complex. Addition of all three individual antisera to the reticulocyte lysate containing the newly synthesized precursor of the iron-sulfur protein revealed that the precursor is only recognized by the antiserum against the iron-sulfur protein and not by the other two antisera (Fig. 1, *lanes 3–5*). These results confirm the specificity of the antisera against the individual subunits and suggest that the antiserum against the intact bc_1 complex does not recognize the iron-sulfur protein by itself.

To investigate the possible assembly of the iron-sulfur protein with other subunits of the bc_1 complex, mitochondria were solubilized with either Triton X-100 or DM after import

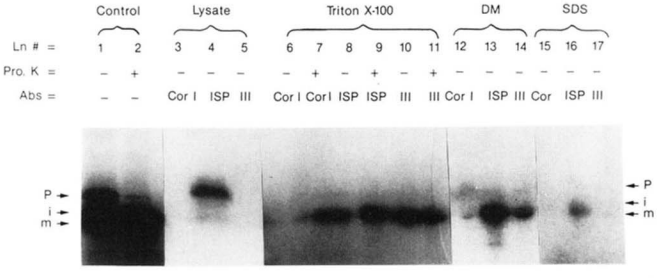

FIG. 1. **Immunoprecipitation of the iron-sulfur protein after import into mitochondria *in vitro*.** The import of the ^{35}S-labeled precursor form of the iron-sulfur protein into isolated mitochondria of the wild-type yeast strain 777-3A was performed as described under "Experimental Procedures." After the import reaction, control mitochondria (*lane 1*) or mitochondria treated with proteinase K (*Pro. K, lane 2*) were analyzed by SDS-PAGE. The reticulocyte lysate containing the ^{35}S-labeled precursor iron-sulfur protein was centrifuged at 100,000 × *g* prior to treatment with specific antisera against the core protein I (*lane 3, Cor I*), the iron-sulfur protein (*lane 4, ISP*), and the cytochrome bc_1 complex (*lane 5, III*), and immunoprecipitates were collected and analyzed as described under "Experimental Procedures." The import reaction was treated with (*lanes 7, 9,* and *11*) or without (*lanes 6, 8, 10,* and *12–17*) proteinase K, and mitochondria were solubilized with either 1% Triton X-100 (*lanes 6–11*), 0.5 mg of DM/mg of protein (*lanes 12–14*), or 3% SDS (*lanes 15–17*), immunoprecipitated by the various antisera as indicated, subjected to SDS-PAGE, and then analyzed by fluorography. The positions of precursor (*p*), intermediate (*i*), and mature (*m*) forms of the iron-sulfur protein are indicated. *Abs*, antibodies.

in vitro. Addition of the antiserum against the bc_1 complex, as well as that against the iron-sulfur protein, to the detergent-solubilized mitochondria resulted in the immunoprecipitation of a radiolabeled mature form of the iron-sulfur protein (Fig. 1, *lanes 6–11*). Similar immunoprecipitates were observed when the import reaction was treated with proteinase K prior to addition of the antisera (Fig. 1, *lanes 7*, *9*, and *11*). By contrast, when mitochondria were solubilized initially with SDS after the import reaction, the antiserum against the bc_1 complex did not immunoprecipitate the mature form of the iron-sulfur protein (Fig. 1, *lane 17*); however, the antiserum against the iron-sulfur protein did immunoprecipitate the mature form from the SDS-solubilized mitochondria (Fig. 1, *lane 16*). Under all conditions studied, the antiserum against core protein I did not immunoprecipitate radiolabeled iron-sulfur protein (Fig. 1, *lanes 3*, *6*, *7*, *12*, and *15*).

These observations suggest that after import and processing, the mature form of the iron-sulfur protein is assembled into the bc_1 complex. Solubilization of the mitochondrial membrane with mild detergents did not dissociate the complex and, hence, the labeled iron-sulfur protein was co-precipitated with the antiserum against the bc_1 complex that recognizes other subunits of the complex. Furthermore, SDS treatment of the mitochondria prior to immunoprecipitation dissociated the imported and assembled iron-sulfur protein from the other subunits of the bc_1 complex. Consequently, the antiserum against the entire bc_1 complex that does not recognize the iron-sulfur protein by itself failed to immunoprecipitate any radiolabeled iron-sulfur protein.

Effects of Processing Inhibitors on Import and Assembly of the Iron-Sulfur Protein into the bc Complex—Our earlier study (32) had indicated that the stepwise processing of the precursor to the intermediate and the mature forms of the iron-sulfur protein was inhibited by increasing concentrations of the metal chelators EDTA and *o*-phenanthroline, as shown in Fig. 2*A*. In addition, when the processing of the precursor to the intermediate form was totally blocked by high concentrations of the inhibitors (Fig. 2*A*, *lane 5*), the precursor form became resistant to exogenous proteinase K, suggesting that translocation of the precursor into the mitochondria had occurred in the absence of processing (Fig. 2*A*, *lane 6*).

Our next approach was to investigate whether the intermediate and the precursor forms internalized when processing was blocked were assembled into the bc_1 complex. The antiserum against the iron-sulfur protein immunoprecipitated the accumulated intermediate form from mitochondria solubilized with Triton X-100 (Fig. 2*B*, *lane 5*); however, the antiserum against the bc_1 complex immunoprecipitated only trace amounts of the intermediate form from the Triton-solubilized mitochondria (Fig. 2*B*, *lane 6*). Similarly, the antiserum against the iron-sulfur protein immunoprecipitated the internalized precursor form, whereas the antiserum against the bc_1 complex immunoprecipitated almost no precursor form from the Triton-solubilized mitochondria (Fig. 2*B*, *lanes 8* and *9*). After solubilization of mitochondria with SDS, only the antiserum against the iron-sulfur protein was able to immunoprecipitate the intermediate form (Fig. 2*C*, *lanes 1–3*). These results confirm that both intermediate and precursor forms of the iron-sulfur protein are recognized by the antiserum against the iron-sulfur protein and suggest that these forms of the iron-sulfur protein are not assembled into the bc_1 complex.

Localization of Intermediate and Mature Forms of the Iron-Sulfur Protein in the Mitochondrial Membrane—The membrane localization of both the intermediate and mature forms of the iron-sulfur protein in mitochondria was determined by

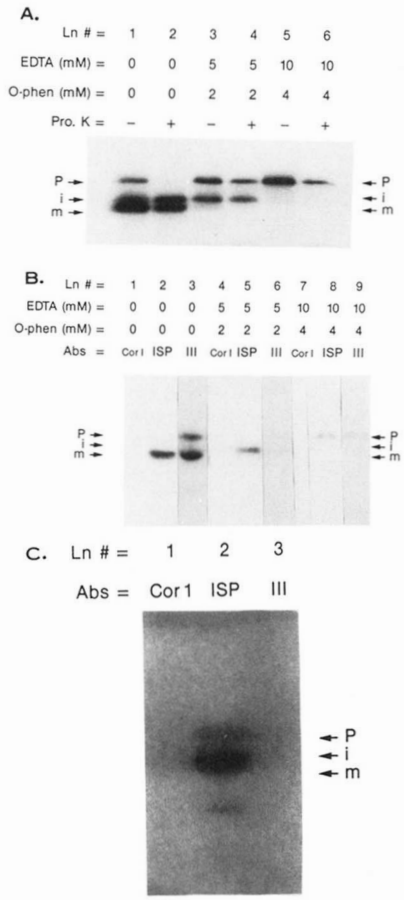

FIG. 2. **Effect of inhibitors on import and assembly of the iron-sulfur protein into the cytochrome bc_1 complex.** *A*, the import of the ^{35}S-labeled precursor form of the iron-sulfur protein into mitochondria isolated from the wild-type yeast strain 777-3A was performed in the presence of inhibitors as described under "Experimental Procedures." *B*, After the import reaction in the presence of 5 mM EDTA and 2 mM *o*-phenanthroline (*lanes 4–6*) or 10 mM EDTA and 4 mM *o*-phenanthroline (*lanes 7–9*), mitochondria were solubilized with 1% Triton X-100, then immunoprecipitated by specific antisera as indicated, and analyzed by SDS-PAGE and fluorography. *C*, experimental conditions were identical with those of *panel B*, except that the import reaction occurred in the presence of 5 mM EDTA and 2 mM *o*-phenanthroline, and mitochondria were treated with 3% SDS prior to immunoprecipitation with specific antisera as indicated. *O-phen*, *O*-phenanthroline. Other abbreviations are as defined in the legend to Fig. 1.

first sonicating the mitochondria after the import reaction, followed by immunoprecipitation with the specific antisera. Fig. 3*A* (*lanes 1* and *2*) shows that the majority of the mature form, as well as the precursor form, is present in the membrane fraction (pellet) after an import reaction *in vitro*. When import was blocked with the chelators EDTA and *o*-phenanthroline, the accumulated intermediate and precursor forms were also largely present in the pellet (*lanes 3* and *4*). Antisera against both the iron-sulfur protein and the bc_1 complex immunoprecipitated the radiolabeled mature form present in the membrane fractions, suggesting that the mature form present in both fractions was assembled with other subunits of the complex (Fig. 3*B*, *lanes 1–4*). In addition, none of intermediate form present in the sonicated pellet after import in the presence of chelators (Fig. 3*A*, *lane 4*) was immunoprecipitated by the antiserum against the bc_1 complex (Fig. 3*B*, *lane 6*); however, some intermediate form was immunoprecipitated from the supernatant with this same antiserum (Fig. 3*B*, *lane 5*). These results, plus those obtained with detergent-

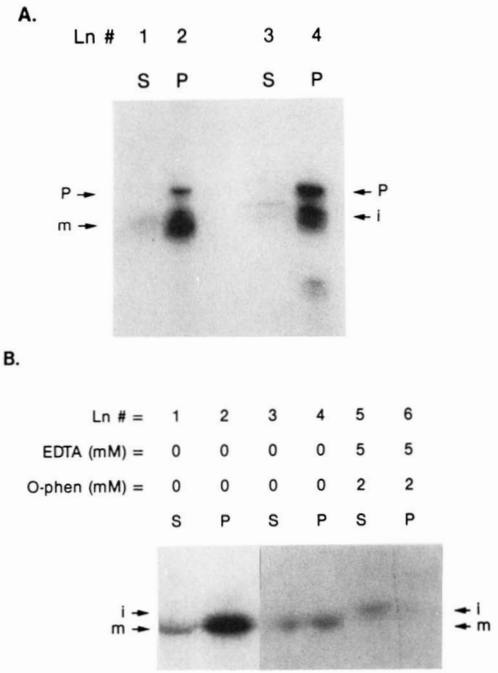

FIG. 3. **Localization of imported forms of the iron-sulfur protein in the mitochondrial membranes after sonication.** Import of the ^{35}S-labeled iron-sulfur protein into isolated mitochondria of the wild-type strain 777-3A in the absence (*lanes 1* and *2*) and presence (*lanes 3* and *4*) of 5 mM EDTA and 2 mM *o*-phenanthroline was performed as described under "Experimental Procedures." After the import reaction, mitochondria were reisolated and resuspended in 0.6 M sorbitol and 20 mM Hepes/KOH (pH 7.4) and sonicated as indicated under "Experimental Procedures." The sonicated mixture was separated into pellet and supernatant fractions by centrifugation at 100,000 × *g* for 1 h. Both pellet and supernatant portions were divided into two equal portions. *A*, one half of both pellet and supernatant was directly analyzed by SDS-PAGE and fluorography. *B*, the remaining half of the pellet was solubilized with 1% Triton X-100, and then both pellet and supernatant were immunoprecipitated with antisera against either the iron-sulfur protein (*lanes 1* and *2*) or the bc_1 complex (*lanes 3–6*), as described under "Experimental Procedures." The immunoprecipitates were analyzed by SDS-PAGE and fluorography. *S*, supernatant, and P, pellet. Other abbreviations are as defined in the legends to Figs. 1 and 2.

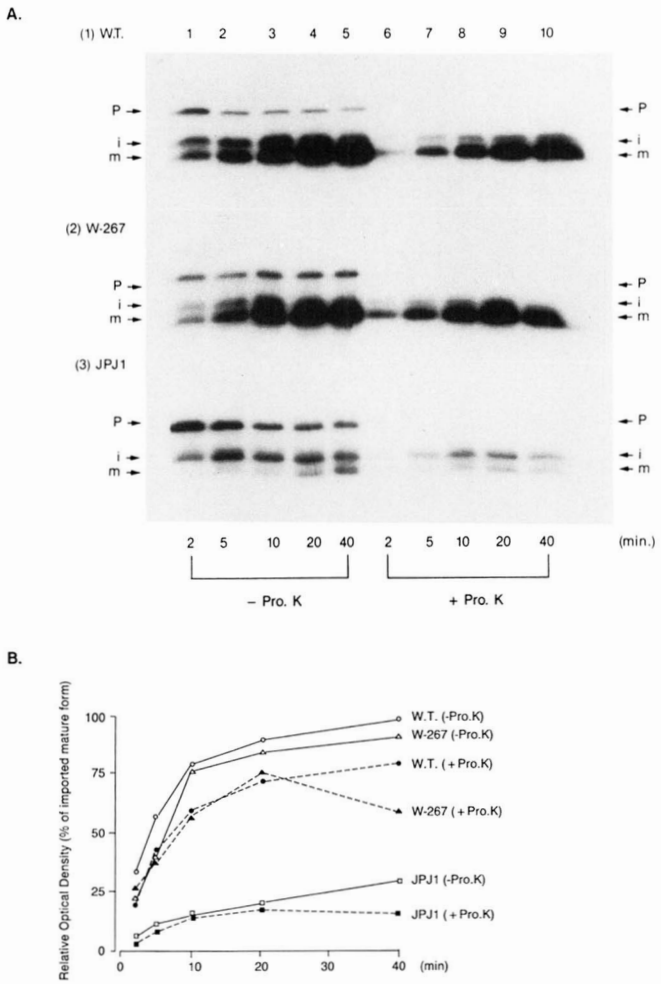

FIG. 4. **Time course of import of iron-sulfur protein into mitochondria.** *A*, the ^{35}S-labeled precursor form of iron-sulfur protein was incubated with isolated yeast mitochondria from the wild-type (777-3A), the cytochrome *b*-deficient (W-267), and the iron-sulfur protein-depleted (JPJ1) strains, as described under "Experimental Procedures." A 400-μl aliquot of the reaction mixture was removed at the times indicated. One half of each 400-μl mixture was directly analyzed by SDS-PAGE (*lanes 1–5*), whereas the remaining half was treated with proteinase K (*lanes 6–10*) and then analyzed by SDS-PAGE. *B*, the bands of the imported mature form of the iron-sulfur protein from the autoradiography of the gels shown in *A* were quantified by laser densitometry. The mature form observed in the wild-type mitochondria at 40 min in the absence of proteinase K was normalized to 100. *W.T.*, wild-type. Other abbreviations used are as defined in the legend to Fig. 1.

solubilized mitochondria (Fig. 2*B*), suggest that the intermediate form does not associate with other subunits of the bc_1 complex in the inner mitochondrial membrane.

Import of the Iron-Sulfur Protein into Mutants Lacking Cytochrome b and the Iron-Sulfur Protein—The importance of the other subunits of the bc_1 complex for proper import and processing of the iron-sulfur protein precursor in mitochondria was investigated in a cytochrome *b*-deficient strain (W-267) and the iron-sulfur protein-depleted strain (JPJ1). In order to maintain a sufficient electrochemical potential ($\Delta\psi$) across the inner membrane of the mutant mitochondria during the import studies, ascorbic acid and TMPD were used as substrates for the cytochrome *c* oxidase complex in the strains lacking a functional bc_1 complex. In addition, phosphoenolpyruvate and pyruvate kinase were added to the import mixture to maintain the ATP levels required for import. The precursor form of the iron-sulfur protein can be imported and processed into mutant mitochondria lacking either cytochrome *b* or the iron-sulfur protein (Fig. 4*A*). Both intermediate and mature forms were resistant to digestion by exogenous proteinase K, whereas the precursor form was sensitive to proteinase K, suggesting that both intermediate and mature forms had been internalized into the mutant mitochondria. For a quantitative comparison of the efficiency of import and processing of the iron-sulfur protein into wild-type and mutant mitochondria, equal aliquots of mitochondria were removed, washed, and subjected to SDS-polyacrylamide gel at various times during the import reaction. Import into mitochondria lacking cytochrome *b* occurred at a rate similar to that of import into the wild-type mitochondria (Fig. 4*B*); however, import into iron-sulfur-depleted mitochondria occurred at a rate only 25% of that into wild-type mitochondria.

The effects of the lack of cytochrome *b* or the iron-sulfur protein on the assembly of the imported iron-sulfur protein into the bc_1 complex was studied using the immunoprecipitation techniques developed for wild-type mitochondria. The mature form of the iron-sulfur protein was immunoprecipitated by the antisera against the iron-sulfur protein and the bc_1 complex in cytochrome *b*-deficient mitochondria solubilized with either Triton X-100 (Fig. 5*A*, *lanes 3* and *4*) or DM (Fig. 5*A*, *lanes 6* and *7*). In addition, a small but significant

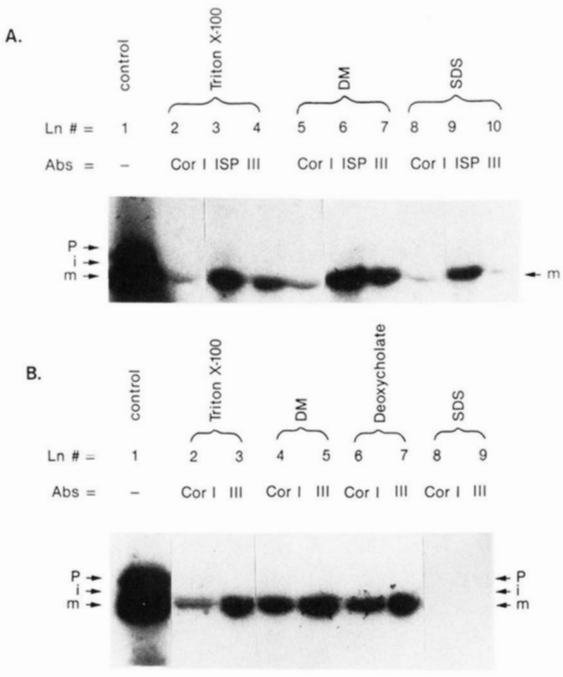

FIG. 5. **Immunoprecipitation of the iron-sulfur protein after import *in vitro* into yeast mutants.** Mitochondria from the cytochrome *b*-deficient (*A*) and the iron-sulfur protein-depleted (*B*) yeast strains were incubated with ^{35}S-labeled iron-sulfur protein as described under "Experimental Procedures." *A*, mitochondria (200 μg) were subjected to SDS-PAGE and analyzed by fluorography (*lane 1*). Mitochondria was also solubilized with 1% Triton X-100 (*lanes 2–4*), 0.6 mg/mg of protein DM (*lanes 5–7*), or 3% SDS (*lanes 8–10*) and immunoprecipitated with the indicated antisera. *B*, mitochondria (200 μg) were subjected to SDS-PAGE (*lane 1*) or solubilized with 1% Triton X-100 (*lanes 2* and *3*), 0.6 mg/mg of protein DM (*lanes 4* and *5*), 0.5 mg/mg of protein deoxycholate (*lanes 6* and *7*), or 3% SDS (*lanes 8* and *9*) and immunoprecipitated by either the antiserum against the core protein or against the bc_1 complex. The abbreviations used are as defined in the legend to Fig. 1.

amount of the mature form was also recognized by the antiserum against core protein I (Fig. 5*A*, *lanes 2* and *5*). Again, after solubilization of mitochondria with SDS prior to immunoprecipitation, only the mature form was significantly immunoprecipitated by the antiserum against the iron-sulfur protein. Trace amounts of labeled iron-sulfur protein were observed in the immunoprecipitates obtained with the antisera against core protein I and the bc_1 complex (*lanes 8–10*). These results indicate that, in the absence of cytochrome *b*, the precursor form of the iron-sulfur protein is imported and processed to the mature form and then assembled with other subunits of the bc_1 complex recognized by the antiserum.

In mitochondria lacking the iron-sulfur protein, the mature form of the radiolabeled iron-sulfur protein was immunoprecipitated by the antiserum against the bc_1 complex (Fig. 5*B*, *lanes 3*, *5*, and *7*), as well as by the antiserum against core protein I (Fig. 5*B*, *lanes 2*, *4*, and *6*); however, antisera against core protein I and the bc_1 complex did not recognize the iron-sulfur protein in mitochondria solubilized with SDS (Fig. 5*B*, *lanes 8* and *9*). These results suggest that the mature form of the iron-sulfur protein is more readily assembled with other subunits of the bc_1 complex in the absence of the iron-sulfur protein in the membrane.

Further investigations of the assembly of the iron-sulfur protein into mitochondria lacking cytochrome *b* and the iron-sulfur protein were performed when the processing reaction was blocked with metal chelators. Addition of 5 mM EDTA and 2 mM *o*-phenanthroline resulted in the accumulation of both intermediate and precursor forms in the wild-type and cytochrome *b*-deficient mitochondria (Fig. 6*A*, *lanes 3* and *11*) and of mainly the precursor form in the iron-sulfur protein-deficient mitochondria (Fig. 6*A*, *lane 7*). Immunoprecipitations with the antiserum against the bc_1 complex indicated that the imported mature form was assembled into the bc_1 complex of the wild-type and cytochrome *b*-depleted mitochondria (Fig. 6*B*, *lanes 1* and *5*); however, both intermediate and mature forms were assembled into the bc_1 complex in the iron-sulfur protein-depleted mitochondria (Fig. 6*B*, *lane 3*). When processing was blocked by the chelators, only small amounts of intermediate and/or precursor forms were recognized by the antiserum against the bc_1 complex (Fig. 6*B*, *lanes 2*, *4*, and *6*). Table I summarizes a quantitation of the three forms of the iron-sulfur protein present in the bc_1 complex of the wild-type and mutant strains. The radiolabeled bands of the three forms of the iron-sulfur protein in the absence of proteinase K observed in Fig. 6, *A* and *B*, were quantified by laser densitometric scanning and are presented as relative optical densities. The percentages indicate the relative amount of that form assembled into the bc_1 complex as indicated by quantitation of the immunoprecipitate. Approximately 16, 13, and 21% of the mature form of the iron-sulfur protein present on the gel was present in the immunoprecipitate of bc_1 complex for the wild-type, cytochrome *b*-deficient,

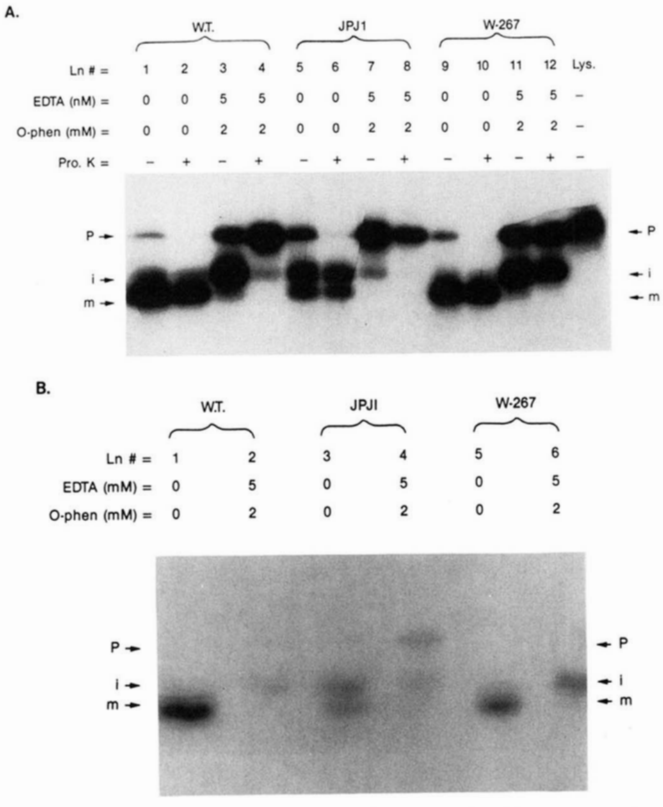

FIG. 6. **Assembly of the iron-sulfur protein after import *in vitro* into mitochondria of wild type and mutants.** *A*, precursor form ^{35}S-labeled iron-sulfur protein was added to isolated mitochondria from the wild-type (*lanes 1–4*), iron-sulfur protein-depleted (*lanes 5–8*), or cytochrome *b*-deficient (*lanes 9–12*) yeast strains in the absence (*lanes 1*, *2*, *5*, *6*, *9* and *10*) or presence (*lanes 3*, *4*, *7*, *8*, *11* and *12*) of 5 mM EDTA and 2 mM *o*-phenanthroline with (*even lanes*) or without (*odd lanes*) added proteinase K. *B*, mitochondria incubated, as indicated, in *lanes 1*, *3*, *5*, *7*, *9* and *11* of Fig. 6*A* were solubilized with 1% Triton X-100, immunoprecipitated by the bc_1 complex antisera, subjected to SDS-PAGE, and analyzed by fluorography. The abbreviations used are as defined in the legends to Figs. 1 and 2.

TABLE I

Assembly of the iron-sulfur protein into mitochondria from wild-type, cytochrome b-deficient, and iron-sulfur protein-depleted yeast stains

Control represents the relative optical density present in the bands of the three forms of the iron-sulfur protein shown in the odd lanes of Fig. 6*A* after quantitation by laser densitometry. The bands representing the immunoprecipitates in Fig. 6*B* were also quantified by laser densitometry. The percentage indicates the value of the immunoprecipitates divided by the total amount in mitochondria after import. ND, not determined.

	Without EDTA and o-phenanthroline		%	With 5 mM EDTA and 2 mM o-phenanthroline		%
	Control	Immunoprecipitate		Control	Immunoprecipitate	
Wild-type						
Precursor	0.186	ND	0	0.550	0.031	6
Intermediate	0.133	ND	0	0.753	0.056	7
Mature	0.800	0.126	16	ND	ND	0
W-267						
Precursor	0.243	ND	0	0.635	0.041	6
Intermediate	0.073	ND	0	0.710	0.071	10
Mature	0.706	0.090	13	ND	ND	0
JPJ1						
Precursor	0.376	ND	0	0.726	0.058	8
Intermediate	0.433	0.071	16	0.120	0.048	40
Mature	0.313	0.067	21	ND	ND	0

and iron-sulfur protein-deficient mitochondria, respectively. In the latter mitochondria, however, 16% of the intermediate form of the iron-sulfur protein was also assembled into the bc_1 complex. In the presence of the chelators, approximately 8, 10, 40% of the intermediate form of the iron-sulfur protein was assembled into the bc_1 complex of mitochondria from wild-type, cytochrome *b*-deficient, and iron-sulfur protein-deficient strains. These results indicate that, in the mutant strain lacking any iron-sulfur protein, the radiolabeled intermediate form can assemble efficiently with other subunits of the bc_1 complex.

DISCUSSION

The results of the present study suggest that the mature form of the iron-sulfur protein can be assembled with the other subunits in the cytochrome bc_1 complex after import *in vitro* into yeast mitochondria. Immunoprecipitates containing the radiolabeled mature form after an import reaction *in vitro* were obtained from detergent-solubilized mitochondria using an antiserum against the intact bc_1 complex. This antiserum did not immunoprecipitate radiolabeled iron-sulfur protein from mitochondria treated with sufficient SDS to disrupt the subunits of the bc_1 complex. In addition, the antiserum against the bc_1 complex did not immunoprecipitate the radiolabeled precursor form of the iron-sulfur protein present in a reticulocyte lysate after translation. It should be noted, however, that an antiserum specific for iron-sulfur protein did immunoprecipitate all forms of the iron-sulfur protein in either SDS-solubilized mitochondria or the reticulocyte lysate. From these observations, we have concluded that the bc_1 complex remains intact after solubilization of mitochondria with gentle detergents such as Triton X-100 and DM. The radiolabeled iron-sulfur protein that has been imported into mitochondria and processed is thus immunoprecipitated with other subunits of the bc_1 complex recognized by the antiserum against the bc_1 complex. Treatment of mitochondria with the strong detergent, SDS, would cause the dissociation of the iron-sulfur protein from the other subunits of the bc_1 complex such that the iron-sulfur protein was not immunoprecipitated with the antiserum against the bc_1 complex.

We next questioned whether the intermediate or precursor forms of the iron-sulfur protein could be assembled into the bc_1 complex in a fashion similar to that of the mature form. Both intermediate and precursor forms of the iron-sulfur protein were accumulated in mitochondria during import *in vitro* by addition of selective concentrations of the protease inhibitors EDTA and *o*-phenanthroline. All three forms of the iron-sulfur protein were immunoprecipitated by the antiserum against the iron-sulfur protein from mitochondria solubilized with various detergents; however, only very low levels of labeled intermediate and precursor forms were observed in the immunoprecipitates obtained with the antiserum against the bc_1 complex. In addition, the intermediate and precursor forms that had been translocated into mitochondria when processing was blocked were present in the membrane fraction obtained after sonication. These observations suggest that the intermediate and precursor forms that accumulate when processing has been blocked may either be inserted into or tightly associated with the inner mitochondrial membrane. Despite this association, the forms of the iron-sulfur protein present in the membrane failed to assemble efficiently with other subunits of the bc_1 complex. These results suggest that processing of the intermediate to the mature form may be a prerequisite for efficient assembly with other subunits of the bc_1 complex. It has been suggested that insertion of the iron-sulfur clusters into the protein is necessary for maintaining the correct conformation prior to translocation back across the inner membrane (37) and assembly with other subunits of the bc_1 complex.

The primary structure of the iron-sulfur protein from *N. crassa* (38), yeast (39), and beef heart (40) has revealed a small hydrophobic stretch of amino acids close to the hydrophilic N terminus and followed by an extensive hydrophilic domain at the C terminus. The hydrophobic portion of the mature protein plus part of the N-terminal presequence may function as the signal for the assembly of the iron-sulfur protein back into the inner membrane. A similar sorting domain on the C-terminal region of the presequence of the cytochrome c_1 was shown to be specific for the mitochondrial inner membrane (41).

The possible role of other subunits of the bc_1 complex in the import and assembly of the iron-sulfur protein was investigated in a mutant strain lacking cytochrome *b* and in a strain in which the nuclear gene for the iron-sulfur protein

had been deleted (33). Previously, it had been reported that mitochondria lacking cytochrome *b* had greatly reduced levels of the iron-sulfur protein plus the low molecular weight subunits (17-, 14-, and 11-kDa proteins) (27, 34, 42). These observations suggested that cytochrome *b* may act as a template in the assembly of certain subunits of the bc_1 complex (34). In the current study, the import and processing of the iron-sulfur protein *in vitro* into mitochondria lacking cytochrome *b* appeared to occur with kinetics similar to those observed in the wild-type mitochondria. The mature form of the iron-sulfur protein also appeared to be assembled with other subunits of the bc_1 complex in the cytochrome *b*-deficient strain as indicated by immunoprecipitates of radiolabeled iron-sulfur protein from mitochondria solubilized with Triton X-100. Other subunits known to be present in the mitochondria from cytochrome *b*-lacking strains include core proteins I and II (27) as well as cytochrome c_1 (42); all of which are recognized by the antiserum against the bc_1 complex (35). By contrast, the rate of import and processing of the iron-sulfur protein into mitochondria lacking any iron-sulfur protein was slower than that into the wild-type and cytochrome *b*-depleted mitochondria. Despite this observation, a significantly greater amount of the imported and processed iron-sulfur protein was assembled with the other subunits of the bc_1 complex than was observed with wild-type and cytochrome *b*-deficient mitochondria. This result was not surprising, since strains lacking the iron-sulfur protein have been shown to contain wild-type levels of the other subunits of the complex (27, 35). Interestingly, the antibody against core protein I was also able to immunoprecipitate radiolabeled iron-sulfur protein in this strain lacking the iron-sulfur protein despite its inability to immunoprecipitate iron-sulfur protein from wild-type mitochondria. In the absence of any iron-sulfur protein, the epitopes of the core protein may be exposed in detergent-solubilized mitochondria and, hence, available to interact with the antiserum against the core protein. These results further substantiate our conclusion that the iron-sulfur protein is assembled into the bc_1 complex after import into mitochondria *in vitro*.

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