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Original Article

Assembly of Deletion Mutants of the Rieske Iron–Sulfur Protein into the Cytochrome bc_1 Complex of Yeast Mitochondria

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The assembly of two deletion mutants of the Rieske iron-sulfur protein into the cytochrome bc_1 complex was investigated after import *in vitro* into mitochondria isolated from a strain of yeast, JPJ1, from which the iron-sulfur protein gene (RIP) had been deleted. The assembly process was investigated by immunoprecipitation of the labeled iron-sulfur protein or the two deletion mutants from detergent-solubilized mitochondria with specific antisera against either the iron-sulfur protein or the bc_1 complex (complex III) [Fu and Beattie (1991). *J. Biol. Chem.* **266,** 16212–16218]. The deletion mutants lacking amino acid residues 55–66 or residues 161–180 were imported into mitochondria *in vitro* and processed to the mature form via an intermediate form. After import *in vitro,* the protein lacking residues 161–180 was not assembled into the complex, suggesting that the region of the iron-sulfur protein containing these residues may be involved in the assembly of the protein into the bc_1 complex; however, the protein lacking residues 55–66 was assembled *in vitro* into the bc_1 complex as effectively as the wild type iron-sulfur protein. Moreover, this mutant protein was present in the mitochondrial membrane fraction obtained from JPJ1 cells transformed with a single-copy plasmid containing the gene for this protein lacking residues 55–66. This deletion mutant protein was also assembled into the bc_1 complex *in vivo,* suggesting that the hydrophobic stretch of amino acids, residues 55–66, is not required for assembly of the iron-sulfur protein into the bc_1 complex; however, this association did not lead to enzymatic activity of the bc_1 complex, as the Rieske FeS cluster was not epr detectable in these mitochondria.

KEY WORDS: Mitochondria; iron–sulfur protein; cytochrome bc_1 complex; complex III; mitochondrial biogenesis.

INTRODUCTION

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 (Hartl *et al.,* 1989; Pfanner and Neupert, 1990). The precursors are generally synthesized containing N-terminal extensions called presequences which are thought to interact with proteinaceous receptors on the cytoplasmic surface of the outer mitochondrial membrane (Baker *et al.,* 1990; Hartl and Neupert, 1990). ATP and cytosolic cofactors, including a heat-shock protein (hsp70), are required to maintain the presequences in an unfolded import-competent conformation (Chirco *et al.,* 1988; Murakami and Mori, 1990). The translocation of the precursor across the mitochondrial membranes requires a membrane potential across the inner membrane plus another hsp70 localized in the mitochondrial matrix (Scherer *et al.,* 1990; Kang *et al.,* 1990). During or after translocation, the precursor may be cleaved by specific mitochondrial protease(s) by either a single or a two-step cleavage event (Hawlitschek *et al.,* 1988;

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Hurt *et al.*, 1985; Fu *et al.*, 1991). The final step in the assembly of the imported proteins into functional complexes in the mitochondria involves the mitochondrial hsp60 which binds the imported protein in the proper conformation in an ATP-dependent process (Ostermann *et al.*, 1989). The mechanism of assembly of imported proteins into functional enzyme complexes within the mitochondria remains to be elucidated. Earlier reports from several laboratories had suggested that the assembly of the different subunits of the cytochrome bc_1 complex[2] into the holoenzyme occurred at different rates in an ordered manner (Sidhu and Beattie, 1983; Crivellone *et al.*, 1988).

The Rieske iron-sulfur protein, a catalytic subunit of the cytochrome bc_1 complex, is anchored on the outer surface of the inner mitochondrial membrane where it protrudes into the intermembrane space (Sidhu *et al.*, 1983; Trumpower and Gennis, 1994). This protein is synthesized as a precursor form with a bipartite presequence which is proteolytically cleaved to the mature form in two steps both *in vitro* (Fu *et al.*, 1990) and *in vivo* (Ostermann *et al.*, 1989). Our laboratory recently reported that the assembly of the iron-sulfur protein into the cytochrome bc_1 complex of yeast mitochondria could be studied *in vitro* by selective immunoprecipitation with antiserum against either the iron-sulfur protein or the intact bc_1 complex (Fu and Beattie, 1991). More recently, chimeric proteins containing parts of the N-terminus of the iron-sulfur protein fused to dihydrofolate reductase and deletion mutants of the iron-sulfur protein were used to investigate the sequences of the precursor required for its import and two-step processing in yeast mitochondria (Japa and Beattie, 1994). Two deletion mutants, one lacking amino acid residues 55–66 and the other lacking amino acid residues 161–180, were efficiently imported into isolated mitochondria *in vitro* where they underwent the identical two cleavages observed after import of the wild type iron-sulfur protein. In the current report, we have used these two deletion mutants to obtain preliminary information about the regions of the iron-sulfur protein that are required for its assembly into the cytochrome bc_1 complex *in vitro* and *in vivo*.

[2] Abbreviations used: bc_1 complex, cytochrome bc_1 complex or complex III (ubiquinol:cytochrome *c* oxidoreductase); RIP, yeast gene encoding the Rieske iron-sulfur protein; rip, mutant gene of the iron-sulfur protein; Rip(Δ55–66), iron-sulfur protein lacking residues 55–66; Rip(Δ161–180), iron-sulfur protein lacking residues 161–180; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Import *in Vitro* of the Wild Type and Deletion Mutants of the Iron-Sulfur Protein into Isolated Mitochondria

The wild type strain, 777-3A, and the strain from which the gene for the iron-sulfur protein had been deleted, JPJ1 (Beckmann *et al.*, 1989), of *S. cerevisiae* were grown and mitochondria isolated as previously described (Fu and Beattie, 1991). The deletion mutants of the iron-sulfur protein were constructed as described (Japa and Beattie, 1994) prior to transcription and translation using an *in vitro* transcription and translation kit supplied by Promega. The import *in vitro* of radiolabeled protein into mitochondria from the JPJ1 cells and immunoprecipitation was performed as previously described (Fu and Beattie, 1991). It should be noted that after the initial solubilization with SDS, the samples were diluted with Triton X-100 or dodecyl maltoside such that the final SDS concentration in the supernatant was less than or equal to 0.2%.

Construction of Deletion Mutants rip(Δ161–180) and rip(Δ55–66) in the Single-Copy Yeast Plasmid PRG415-RIP and Transformation of Yeast Cells

The Hind III/Sac I DNA fragments containing the deletions in the iron-sulfur protein gene (RIP) were gel-purified after restriction digestion of the expression vector pSP64-rip(Δ161–180 and pSP64-rip(Δ55–66) (Japa and Beattie, 1994). This fragment was subcloned into a single-copy yeast plasmid PRG415-RIP previously digested with Hind III/Sac I. Intact JPJ1 yeast cells were transformed with the single copy plasmid containing the gene for the wild type iron-sulfur protein or the deletion mutants, rip(Δ161–180) and rip(Δ55–66), using the alkali cation procedure with lithium acetate (Ito *et al.*, 1983; Dunn *et al.*, 1984). The yeast cells transformed with the single-copy plasmid were selected by plating on a medium lacking uracil.

Pulse Labeling and Assembly *in Vivo*

JPJ1 cells were transformed with the single-copy plasmid containing rip (Δ161–180) and grown aerobically to late logarithmic phase at 30°C in a medium containing yeast nitrogen base without amino acids

(6.7 g/liter; Difco), adenine (80 mg/liter), histidine (20 mg/liter), tryptophan (20 mg/liter), pH 5.0, and glucose (20 g/liter) as the carbon source (Gasser *et al.*, 1982). The wild-type strain, W303-1A, was grown aerobically to late logarithmic phase at 30°C in the same medium supplemented with leucine (80 mg/liter) and uracil (20 mg/liter). The yeast cells were harvested by centrifugation at 1500 × *g* for 10 min, washed once with distilled water, resuspended at a concentration of 200 mg of cells, wet weight/ml, in labeling medium, and incubated at 30°C for 5 min prior to labeling by a modification of our previous procedure (Sidhu and Beattie, 1983). The labeling medium was identical to the growth medium except that the concentrations of nitrogen base without amino acids, adenine, uracil, leucine, tryptophan, histidine, and glucose were decreased 75% compared to the growth medium. The cells were then pulse-labeled with 200 μCi of [^{35}S]methionine/ml of labeling medium. At the indicated times, the radioactive label was chased by the addition of 10 mM unlabeled methionine. An aliqout containing 200 mg of cells was removed at the indicated times and broken immediately with glass beads. The protein was then precipitated by the addition of trichloroacetic acid (w/v, final concentration, 20%), centrifuged at 5000 × *g* for 10 min, and washed 3 times with the labeling medium minus the radioactive [^{35}S]-methionine. The washed protein pellet was dissolved in 400 μl of sample buffer containing 1% Triton X-100, 0,15 M Tris, pH-7.4, 5 mM EDTA, and 0.1% bromophenol blue. Following a 1-h incubation at room temperature, the solubilized extracts were centrifuged at 100,000 × *g* for 30 min to remove insoluble material. The supernatants were then mixed with the antisera against either the iron-sulfur protein or the intact cytochrome bc_1 complex (complex III) and incubated at 4°C with gentle shaking. Sepharose bound protein A (10–12 mg, dry weight), pre-swollen in 100 μl of a solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2mM 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 at 10,000 × *g* and washed twice with 50 mM Tris-HCl, pH-7.4, 150 mM NaCl, 2 mM EDTA, and 0.2% Triton X-100 and twice 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 10,000 × *g* for 5 min and the supernatants were subjected to SDS-PAGE. The gels were fixed with 10% acetic acid and 40% ethanol for 30 min and then stained with 0.1% Coomassie blue (G250) in 10% acetic acid for 20 min. The gels were destained overnight in 10% acetic acid solution and then sliced into 2-mm slices which were digested for 16 h at 60°C with 1 ml of 30% hydrogen peroxide. The digested samples were then cooled to 4°C and dissolved in 10 ml of scintillation fluid and counted in an LKB Rackbeta scintillation counter.

EPR Spectroscopy

Mitochondria for EPR studies were isolated from the wild type cells (W303-1A), from the strain lacking RIP(JPJ1), and from JPJ1 transformed with the single-copy plasmid containing rip (Δ55–66). The cells were broken with glass beads and mitochondria isolated by differential centrifugation (Kim and Beattie, 1973). The mitochondrial suspension was reduced with dithionite and the EPR spectra recorded as described previously (Lin *et al.*, 1983).

Materials and Miscellaneous Methods

L-[^{35}S]methionine (1200 Ci/mmol) was obtained from ICN Biomedicals, Inc. Nuclease-treated rabbit reticulocyte lysate and an amino acid mixture minus methionine were purchased from Promega. Prestained molecular weight markers were purchased from Diversified Biotech. HEPES and EDTA were from Sigma Chemical Company.

SDS-PAGE was performed and scanned by laser densitometry as previously described (Fu and Beattie, 1991). Immunoblots were performed using the ECL Western blotting system (Kleijmeer *et al.*, 1992).

RESULTS

Assembly of the Iron-Sulfur Protein into the Cytochrome bc_1 Complex

Several years ago, we reported that the mature form of the iron-sulfur protein was assembled into the cytochrome bc_1 complex after import *in vitro* into yeast mitochondria (Fu and Beattie, 1991). This conclusion was based on the observation that the labeled mature form of the iron-sulfur protein after import was immunoprecipitated from mitochondria solubilized with

either Triton X-100 or dodecyl-maltoside using an antiserum against the intact bc_1 complex (complex III). The complex III antiserum used in these experiments recognized core protein I, core protein II, cytochrome *b*, and cytochrome c_1 as well as subunits 6 and 7 in immunoblots of the bc_1 complex, *but did not recognize the iron-sulfur protein.* It should also be noted that mitochondrial membranes solubilized with these concentrations of detergents retained ubiquinol:cytochrome *c* reductase activity, suggesting that the bc_1 complex was intact under these conditions. The antiserum against the bc_1 complex, or complex III, did not immunoprecipitate the precursor form of the labeled iron-sulfur protein from reticulocyte lysates (Fig. 1) or from mitochondria treated with sufficient SDS after import to dissociate the subunits of the complex prior to dilution with Triton X-100 or dodecyl maltoside and incubation with the antiserum (Fig. 2). The antiserum against the iron-sulfur protein (ISP) did immunoprecipitate the labeled precursor form of the iron-sulfur protein from the reticulocyte lysate and from mitochondria after the import reaction *in vitro* even after solubilization with SDS (Fig. 2). These results have suggested that the labeled iron-sulfur protein observed in the immunoprecipitates obtained with the complex III antiserum had been co-immunoprecipitated with other subunits of the bc_1 complex recognized by the

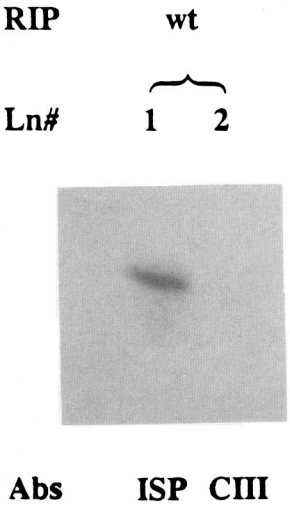

Fig. 1. Specificity of antibodies used in the immunoprecipitation of the iron-sulfur protein from reticulocyte lysates. The precursor form of the iron-sulfur protein was labeled with [^{35}S]methionine by transcription and translation in the reticulocyte lysate. Ribosomes were removed by centrifugation and the supernatant treated with either the antibody against the iron-sulfur protein (ISP) or against the bc_1 complex (CIII) and analyzed by SDS-PAGE and fluorography. Abs, antibodies.

complex III antiserum, an indication that the imported iron-sulfur protein had been assembled into the bc_1 complex (Fu and Beattie, 1991).

Figure 2 confirms these observations, as addition of antiserum against the iron-sulfur protein to mitochondria solubilized with SDS prior to dilution with Triton X-100 immunoprecipitated both the precursor and mature forms of the labeled iron-sulfur protein; however, addition of the antiserum against complex III to the SDS-solubilized mitochondria *did* not immunoprecipitate labeled iron-sulfur protein (Fig. 2, lanes 1,2). After solubilization of mitochondria with Triton X-100, however, addition of antisera against both the iron-sulfur protein and complex III immunoprecipitated the labeled mature form of the iron-sulfur protein (Fig. 2, lanes 3,4), suggesting that the mature form of the iron-sulfur protein after import into yeast mitochondria *in vitro* has been assembled with the other subunits of the bc_1 complex which are recognized by the complex III antiserum. It should be noted that identical results were obtained when the mitochondria were solubilized with another detergent, dodecyl maltoside, prior to immunoprecipitation. We also suggest that the presence of the precursor form of the iron-sulfur protein in the immunoprecipitates obtained after the initial solubilization with SDS but not with Triton X-100 with the antiserum against the iron-sulfur protein results because the more powerful detergent, SDS, can solubilize the precursor form from the outer membrane.

To calculate the efficiency of import of the iron-sulfur protein into yeast mitochondria, the radioactivity in the immunoprecipitate obtained with the complex III antiserum from mitochondria solubilized with Triton X-100 was determined by laser densitometry. This value was divided by the radioactivity in the immunoprecipitate obtained with the RIP antiserum from Triton X-100 solubilized mitochondria which was assumed to represent the total amount of the iron-sulfur protein imported into mitochondria (Table I).

The individual steps involved in processing the iron-sulfur protein from the precursor to the intermediate form and from the intermediate to the mature form are selectively inhibited by increasing concentrations of the metal chelators EDTA and *O*-phenanthroline (Ramabadran and Beattie, 1992). Using this protocol, we attempted to show whether the precursor and the intermediate forms of the iron-sulfur protein could assemble into the bc_1 complex when processing was blocked. In these studies, the inhibitors, EDTA and *O*-phenanthroline, were used at concentrations which

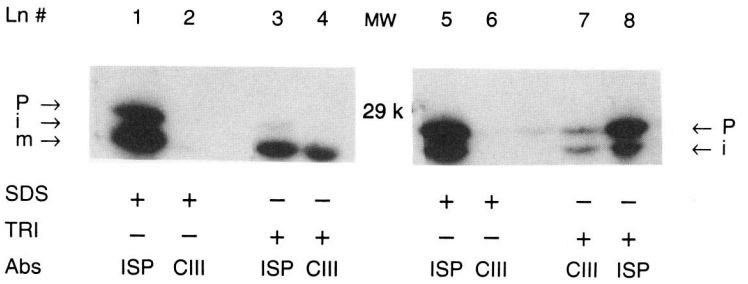

Fig. 2. Assembly of the iron-sulfur protein into the cytochrome bc_1 complex after import *in vitro* into mitochondria of JPJ1 cells in the presence and absence of inhibitors. Isolated mitochondria from JPJI cells (100 μg of protein] were preincubated in a 200-μl reaction mixture containing 0.6 M sorbitol, 20 mM Hepes/KOH buffer (pH 7.4), 40 mM KCl, 1 mM DTT, 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 for 5 min at 27°C (lanes 1–4). The inhibitors EDTA (5 mM) and *O*-phenanthroline (1 mM) were included in the incubation mixture in lanes 5–8. Translation mixture (5 μl) containing the radiolabeled precursor was added to the reaction mixture and the incubation continued for 30 min. After the import reaction, mitochondria were solubilized with either Triton (TRI) X-100 or SDS, immunoprecipitated with the antiserum against either the iron-sulfur protein (ISP) or complex III (CIII), separated by SDS-PAGE, and analyzed by fluorography. The positions of the precursor (p), intermediate (i), and mature (m) forms of the iron-sulfur protein are indicated. Abs, antibodies.

Table I. Assembly of the Iron-Sulfur Protein and Its Deletion Mutants rip(Δ55–66) and rip(Δ161–180) into the Cytochrome bc_1 *in Vitro*[a]

	Assembly
Iron-sulfur protein	72 ± 8%
Iron-sulfur protein plus inhibitors 8%	
Deletion mutant (Δ55–66)	75 ± 5%
Deletion mutant (Δ161–181)	12%

[a] The percent assembly was calculated by dividing the radioactivity present in the immunoprecipitate obtained with the antiserum against compled III by the radioactivity present in the immunoprecipitate obtained with the antibody against the iron-sulfur protein from mitochondria solubilized with Triton X-100. The radioactivity was determined by laser densitometry. The values reflect the mean ±SEM obtained from 3–6 individual experiments or the average of 2 experiments.

partially block the processing of the precursor to the intermediate form but completely block the processing of the intermediate to the mature form. Previously, we had reported that when processing of the iron-sulfur protein was blocked, the precursor and intermediate forms were imported into a protease-resistant compartment of the mitochondria (Ramabadran and Beattie, 1992). Under these conditions (Fig. 2, lane 7), both the precursor and the intermediate forms were not appreciably assembled into the bc_1 complex when compared to the mature form after import in the absence of inhibitors (Fig. 2, lane 4).

Assembly of the Deletion Mutants Rip(Δ55–66) and Rip(Δ161–180) of the Iron-Sulfur Protein

In the membrane-bound cytochrome bc_1 complex, the iron-sulfur protein is exposed on the exterior face of the mitochondrial inner membrane where it is accessible to digestion by exogenous proteases (Sidhu *et al.*, 1983; Brandt and Trumpower, 1994). A hydrophobic domain of amino acids, residues 55–66, of the iron-sulfur protein near the amino terminus has been suggested to anchor the protein to the inner membrane (Gonzalez-Halphen *et al.*, 1988). Deletion of this hydrophobic stretch of amino acids in RIP did not affect the processing of the imported precursor to the mature protein (Japa and Beattie, 1994). Similarly, the deletion mutant Rip(Δ161–180) which lacks the 20 amino acid sequence involved in binding the FeS cluster to the protein was imported and efficiently processed to both the intermediate and the mature forms after import *in vitro* (Japa and Beattie, 1994).

Before studying the assembly of these two mutant proteins into the bc_1 complex, it was necessary to establish that the antiserum against the iron-sulfur protein could recognize these proteins. The reticulocyte

lysate containing the newly synthesized precursor forms of the iron-sulfur protein and the two deletion mutants, RipΔ55–66) and Rip(Δ161–181), was initially solubilized with either Triton X-100 or SDS prior to incubation with the antibody against the iron-sulfur protein. Immunoprecipitates of the precursor forms of the wild type and the two deletion mutants were clearly visible after SDS-PAGE (Fig. 3), suggesting that the antiserum against the iron-sulfur protein recognizes these proteins whether Triton X-100 or SDS was used for the initial solubilization.

The assembly of Rip(Δ55–66) and Rip(Δ161–180) into the cytochrome bc_1 complex *in vitro* was studied after import into yeast mitochondria. The mitochondria after the import reaction were solubilized with SDS or Triton X-100 prior to incubation with and immunoprecipitation with the antisera against either the iron-sulfur protein or complex III. Figure 4A indicates that the deletion mutant lacking the hydrophobic stretch of amino acids, 55–66, of the iron-sulfur protein was assembled into the bc_1 complex after import *in vitro* as efficiently as the wild type based on the comparable immunoprecipitates obtained with either antibody after solubilization of the mitochondria with Triton X-100 (Fig. 4A, lanes, 4,5 and Table I). By contrast, only the antiserum against the iron-sulfur protein immunoprecipitated radiolabeled protein, both the precursor and mature forms of RIP(Δ55–66) after the initial solubilization with SDS (Fig. 4A, lanes 2,3). These results suggest that the stretch of hydrophobic amino acids, residues 55–66, is not essential for the interaction of the iron-sulfur protein with other subunits of the bc_1 complex during assembly after import *in vitro*. Figure 4B indicates, however, that the deletion mutant, Rip(Δ161–180) was not assembled into the

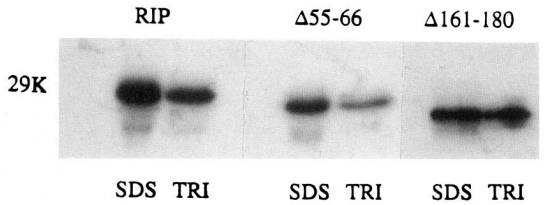

Fig. 3. Immunoprecipitation of the mutant proteins by the antiserum against the iron-sulfur protein. Transcription/translation of the iron-sulfur protein (RIP) and the two deletion mutants rip(Δ55–66) and rip(Δ161–181) in the reticulocyte lysate was performed as described in the experimental procedures. After centrifugation to remove ribosomes, the supernatants were solubilized with either SDS or Triton X-100 (TRI) prior to incubation with the iron-sulfur protein antibody. The immunoprecipitates were separated by SDS-PAGE followed by fluorography.

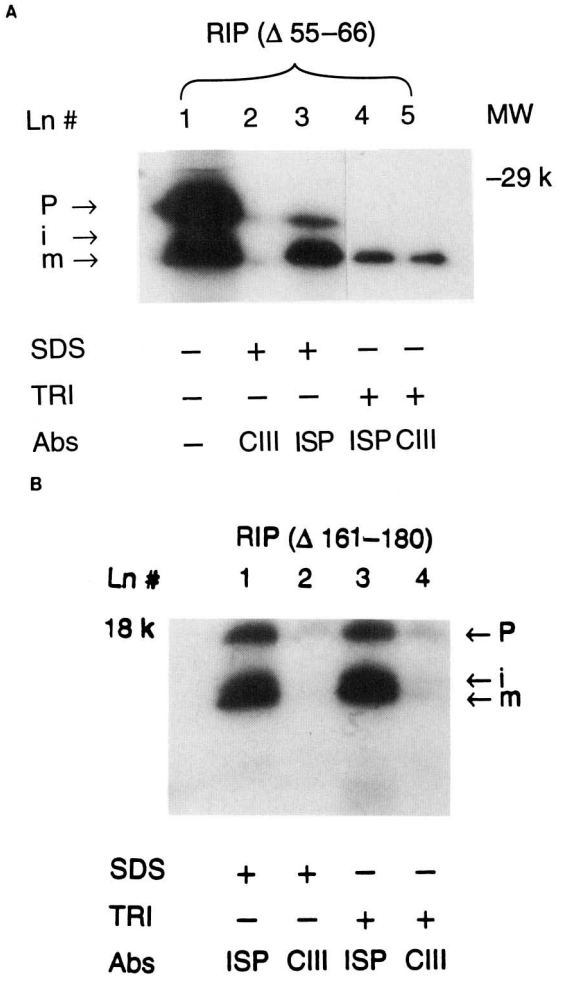

Fig. 4. Assembly of the deletion mutants of the iron-sulfur protein, Rip(Δ55–66), and Rip(Δ161–180), into the bc_1 complex after import *in vitro* into mitochondria of JPJ1 cells. Isolated mitochondria were incubated with the radiolabeled precursor to Rip(Δ55–66); A, lanes 1–4, or Rip(Δ161–180), B, lanes 1–4, prior to immunoprecipitation and analysis as described in the legend to Fig. 1. The positions of the precursor (p), intermediate (i), and mature (m) forms of the deletion mutants are indicated. Abs, antibodies; iron-sulfur protein (ISP), complex III (CIII).

bc_1 complex as only traces of radioactivity were observed after immunoprecipitation with the antibody against complex III compared to that obtained with the antibody against the iron-sulfur protein. These results suggest that the 20 amino acid sequence containing the residues involved in binding the FeS cluster to the protein may be essential for the interaction of the iron-sulfur protein with other proteins of the complex during the assembly process. Alternatively, this deletion may cause significant changes in the conformation of the iron-sulfur protein such that assembly is blocked.

In Vivo Transformation of JPJ1 with Plasmids Containing the Genes for RIP, rip(Δ55–66), and rip(Δ161–180)

The results presented above indicated that both iron-sulfur protein and Rip(Δ55–66) were efficiently assembled into the bc_1 complex after import *in vitro* into yeast mitochondria lacking the iron-sulfur protein (strain JPJ1) while Rip(Δ161–180) was not assembled into the bc_1 complex *in vitro*. To establish whether the results obtained *in vitro* provide an accurate reflection of processes occurring *in vivo*, JPJ1 cells were transformed with a single-copy plasmid containing the genes for either the iron-sulfur protein (RIP), Rip(Δ55–66) or Rip(Δ161–180). Transformation of JPJ1 with the RIP gene for the wild type iron-sulfur protein restored the ability of JPJ1 to grow on glycerol, indicating that the iron-sulfur protein had been synthesized and assembled into a functional bc_1 complex *in vivo*. Transformation of JPJ1 with the plasmids containing the two deletion mutants of the iron-sulfur protein, however, did not restore respiratory competence to these cells.

We next investigated whether the mutant forms of the iron-sulfur protein were expressed in these transformed cells. An immunoblot of total yeast cell protein with the RIP antiserum revealed the presence of the mature forms of both the iron-sulfur protein and the deletion mutant, Rip(Δ55–66), in yeast cells (Fig. 5, lanes 1 and 2). In addition, both the precursor and mature forms of the Rip(Δ 55–66) mutant were present in the mitochondria isolated from these cells (Fig. 5, lane 6). Subfractionation of the mitochondria by sonication revealed that the mature form of Rip(Δ55–66) was associated with the membrane fraction (Fig. 5, lane 5), while the precursor form was present in the supernatant fraction (Fig. 5, lanes 3 and 4). The association of the mature form of the mutant with the mitochondrial membrane *in vivo* provides further evidence for the conclusion that amino acid residues 55–66 are not required for association of RIP with the membrane.

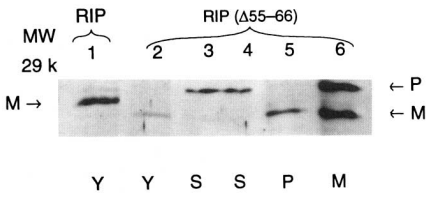

Fig. 5. Immunoblot analysis of JPJ1 cells transformed with the single-copy plasmid containing the gene for either the iron-sulfur protein (RIP) or the deletion mutant [rip(Δ55–66)]. Yeast cells were harvested, broken with glass beads, and then treated with 20% trichloroacetic acid (w/v, final concentration) prior to SDS-PAGE and immunoblotting as described in Materials and Methods (lanes 1 and 2). Mitochondria from JPJ1 cells transformed with rip(Δ55–66) were isolated using the zymolyase method (lane 6), and sonicated prior to centrifugation at 100,000 × *g* for 30 min. The supernatant fraction after sonication (lanes 3 and 4) and the pellet containing mitochondrial membranes (lane 5) were analyzed. Y, yeast cells, S, supernatant, and P, pellet.

By contrast, protein corresponding to the mutant Rip(Δ161–180) lacking the amino acids that bind the FeS clusters was not detectable in immunoblots of intact JPJ1 cells transformed with the rip(Δ161–180) gene (data not shown). The ability of the JPJ1 cells to grow on a medium lacking uracil suggested that the transformation with the single-copy plasmid containing rip(Δ161–180) had been successful. Pulse labeling of both wild type and mutant yeast cells for 15 min followed by immunoprecipitation with the antiserum against the iron-sulfur protein revealed the presence of the mature form in the wild type cells; however, the mutant Rip(Δ161–180) could not be detected (data not shown). The lack of a mature form of the deleted protein may reflect either a lack of synthesis or alternatively a rapid degradation of the protein which is not assembled with other subunits of the bc_1 complex.

Assembly of the Iron-Sulfur Protein into the bc_1 Complex *in Vivo*

The presence of the mature form of Rip(Δ55–66) in the mitochondrial membranes of the transformed cells suggested that assembly into the bc_1 complex may have occurred *in vivo* as was observed *in vitro* (Fig. 4). To test this possibility, yeast cells were pulse-labeled with [^{35}S]methionine and then immunoprecipitated with both the antisera against the iron-sulfur protein and complex III. Labeled mature iron-sulfur protein was observed in immunoprecipitates obtained with the iron-sulfur protein antiserum in both wild type yeast cells (W303-1A) and JPJ1 cells transformed with rip(Δ55–66) (Fig. 6A and C). It should be noted that the molecular weight of the mutant protein is approximately 1 kDa less than that of the wild type protein as anticipated with a deletion of 11 amino acids from the protein. The amount of immunoprecipitable iron-sulfur protein increased after a 25 min chase with cold methionine (Fig. 6A and C). The complex III antiserum immunoprecipitated several subunits of the bc_1 com-

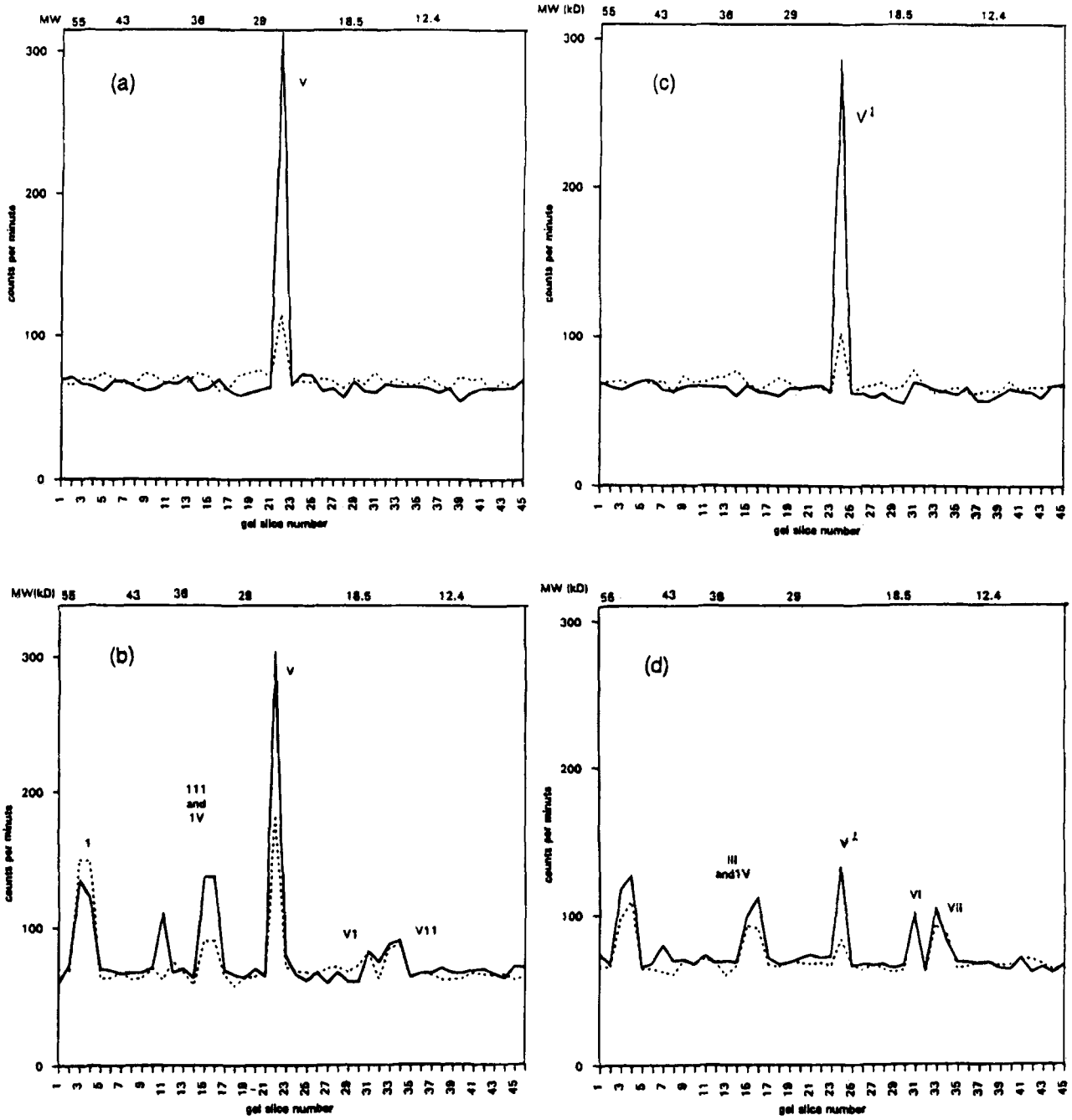

Fig. 6. Assembly of the iron-sulfur protein *in vivo* into the bc_1 complex in wild type and JPJ1 cells expressing Rip(Δ55–66). Wild type cells, W303-1A (A and B) and JPJ1 cells transformed with a single-copy plasmid PRG415 containing rip(Δ55–66) (C and D) were pulse-labeled with [^{35}S]-methionine for 3 min. An aliquot of cells was removed immediately (. . .). Unlabeled methionine was added and the incubation continued for 25 min prior to isolation of the cells (——). The cells were then solubilized with 1% Triton X-100 and immunoprecipitated with the antiserum against the iron-sulfur protein (A and C) or the antiserum against complex III (B and D). After SDS-PAGE, the gel was sliced into 2 mm thick pieces, digested with hydrogen peroxide, and counted. (. . .) radioactivity observed after the initial 3 min pulse; (———) radioactivity after the 25 min chase.

plex including core 1 (subunit I), cytochrome *b* and cytochrome c_1 (subunits III and IV are not separated under these gel conditions), subunit VI, subunit VII, and mature RIP (subunit V) in the wild type and mutant iron-sulfur protein (subunit V') in the transformed JPJ1 cells (Fig. 6B and D). Subunit II was not labeled by the [^{35}S]methionine, because this polypeptide does not contain methionine residues (Sidhu and Beattie, 1983). The presence in the immunoprecipitates of the mutant iron-sulfur protein with the other subunits of the bc_1 complex suggests that the mutant protein lacking amino acids 55–66 has been associated with the other subunits of the bc, complex *in vivo* (Fig. 6D).

The inability of the Rip(Δ55–66) protein to restore respiratory competence to JPJ1 despite its assembly into the bc_1 complex *in vivo* prompted us to investigate whether the Rieske FeS cluster was present on the mutant protein. EPR spectroscopy of dithionite-reduced mitochondria prepared from the wild type (W3031-A), JPJ1, and from JPJ1 cells transformed with rip(Δ55–66) was used to establish the presence or absence of the FeS cluster in these cells. The EPR signal of the FeS cluster is evident in mitochondria from the wild type cells, W303-1A, at its main signal at $g_z = 2.03$ and with a negative peak at $g_y = 1.89$ Lin *et al.*, 1983). The g_x signal around $g_x = 1.80$ cannot be distinguished because of the low signal-to-noise ratio (Fig. 7). By contrast, no EPR signals due to the FeS cluster were observed in mitochondria from either JPJ1 cells or JPJ1 cells transformed with rip(Δ55–66).

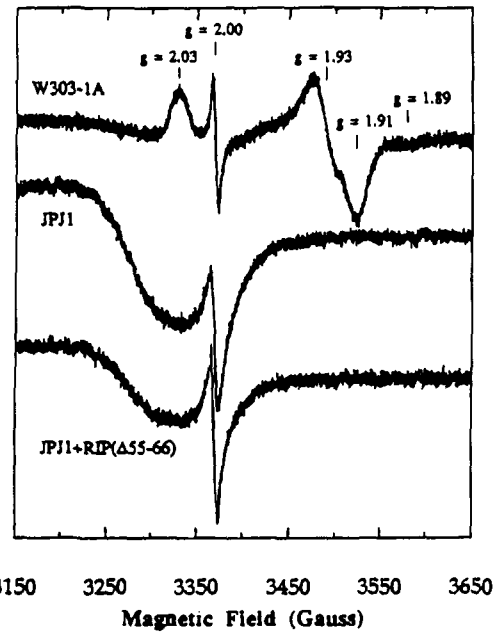

Fig. 7. EPR spectra of iron-sulfur clusters of the iron-sulfur protein in the bc_1 complex. Mitochondrial suspensions (48 mg/ml) were reduced with a few crystals of dithionite before freezing at 15°K. Upper trace, mitochondria from wild-type, W303-1A, cells; middle trace, mitochondria from JPJ1 cells; bottom trace, mitochondria from the JPJ1 cells transformed with a single-copy plasmid PRG415 rip(Δ55n66).

DISCUSSION

In the current study, two deletion mutants of the iron-sulfur protein have been used to obtain preliminary evidence as to which regions of this protein are involved in its assembly *in vitro* into the cytochrome bc_1 complex of yeast mitochondria. The results obtained suggest that the deletion mutant lacking amino acid residues 55–66 located in a hydrophobic region of the iron-sulfur protein was assembled into the bc_1 complex as efficiently as the wild type *in vitro* (Fig. 4A and Table I). Moreover, this mutant protein was also assembled *in vivo* into the bc_1 complex of JPJ1 transformed with rip(Δ55–66). Amino acid residues 55–66 of the iron-sulfur protein are included in a hydrophobic region which had been previously suggested to anchor this protein to the inner membrane either by spanning the membrane (Brandt and Trumpower, 1994; Trumpower and Gennis, 1994) or by binding to the membrane by hydrophobic interactions (Gonzalez-Halphen *et al.*, 1991). The results of the current study, however, suggest that this hydrophobic region of the iron-sulfur protein is not required for its assembly into the bc_1 complex. Despite the assembly of this deletion mutant lacking amino acid residues 55–66 into the bc_1 complex *in vivo*, no EPR signals corresponding to the Rieske FeS cluster could be detected in these cells. We suggest that the mutant iron-sulfur protein lacking these residues may interact with the other subunits of the bc_1 complex in the assembly process; however, the deletion mutant may not assume the correct conformation required for insertion of the FeS cluster.

By contrast, another deletion mutant of the iron-sulfur protein lacking 20 amino acid residues, Rip(Δ161–180), which underwent the two expected processing events after import *in vitro* (Japa and Beattie, 1994), was assembled very inefficiently into the bc_1 complex (Fig. 4B). These results suggest that the region of the iron-sulfur protein containing amino acid residues 161–180 may be involved in the interaction of the iron-sulfur protein with other subunits of the bc_1 complex during the assembly process (Sidhu and

Beattie, 1983; Crivellone *et al.*, 1988). Alternately, such a large deletion in the iron-sulfur protein may result in the protein adopting a conformation incapable of assembly with the other subunits of the complex. Consequently, the mutant protein which has not been assembled into the bc_1 complex may, in turn, become susceptible to proteolytic digestion by intracellular proteases.

The results of the current study also suggest that the assembly process observed *in vitro* is an accurate reflection of events occurring *in vivo*. As discussed above, the iron-sulfur protein lacking amino acid residues 55–66 was assembled into the bc_1 complex as efficiently as the wild type iron-sulfur protein both *in vitro* and *in vivo*. The deletion mutant Rip(Δ161–180) did not assemble significantly into the bc_1 complex *in vitro* and could not be detected *in vivo* either by immunoblotting of JPJ1 yeast cells transformed with rip(Δ161–180) or by immunoprecipitation with the iron sulfur protein antiserum after a 15-min pulse label of these cells. Currently, we are investigating other deletion mutants of the ISP to further characterize the regions of the protein required for its assembly into the bc_1 complex.

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REFERENCES

Baker, K. P., Schaniel, A., Vestweber, D., and Schatz, G. (1990). *Nature* **348**, 605–609.

Beckmann, J. D., Ljungdhal, and Trumpower, B. L. (1989). *J. Biol. Chem.* **264**, 3713–3722.

Brandt, U., and Trumpower, B. (1994). *Crit. Rev. Biochem. Mol. Biol.* **29**, 165–197.

Chirco, W. J., Waters, G., and Blobel, G. (1988). *Nature* **332**, 805–811.

Crivellone, D. M., Wu, M., and Tzagoloff, A. (1988). *J. Biol. Chem.* **263**, 14323–14357.

Dunn, B., Szauter, P., Pardue, M. L., and Szostak, J. (1984). *Cell* **39**, 191–201.

Fu, W., and Beattie, D. S. (1991). *J. Biol. Chem.* **266**, 16212–16218.

Fu, W., Japa, S., and Beattie, D. S., (1990). *J. Biol. Chem.* **265**, 16541–16547.

Gasser, S. M., Ohashi, G., Daum, P., Bohini, J., Gibson, G., Reid, T., Yonetani, R., and Schatz, G. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 267–271.

Gonzalez-Halphen, D., Lindorfer, M. A., and Capaldi, R. A. (1988). *Biochemistry* **27**, 7021–7031.

Gonazalez-Halphen, D., Vazquez-Acevedo, M., and Garcia-Ponce, B. (1991). *J. Biol. Chem.* **266**, 3870–3876.

Hartl, F.-U., and Neupert, W. (1990). *Science* **247**, 930–938.

Hartl, F.-U., Pfanner, N., Nicholson, D. W., and Neupert, W. (1989). *Biochim. Biophys. Acta* **988**, 1–45.

Hawlitschek, G., Schneider, H., Schimdt, B., Tropschug, M., Hartl, F.-U., and Neupert, W. (1988). *Cell* **53**, 795–806.

Hurt, E. C., Pesold-Hurt, B., Suda, K., Oppliger, W., and Schatz, G. (1985). *EMBO J.* **4**, 2061–2068.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). *J. Bacteriol* **153**, 163–168.

Japa, S., and Beattie, D. S. (1994). *Arch. Biochem. Biophys.* **312**, 414–420.

Kang, P. J., Ostermann, J., Schilling, J., Neupert, W., Craig, E. A., and Pfanner, N. (1990). *Nature* **348**, 137–143.

Kim, I. C., and Beattie, D. S. (1973). *Eur. J. Biochem.* **36**, 509–518.

Kleijmeer, M. J., Kelly, A., Genze, H. J., Slot, J. W., Townsend, A., and Trowsdale, J. (1992). *Nature* **357**, 342–344.

Lin, C. I. P., Ohnishi, T., Clejan, L., and Beattie, D. S. (1983). *Eur. J. Biochem.* **137**, 179–183.

Murakami, M., and Mori, M. (1990). *EMBO J.* **9**, 3201–3208.

Ostermann, J., Horwich, A. L., Neupert. W., and Hartl, F.-U. (1989). *Nature* **341**, 125–130.

Pfanner, N., and Neupert, W. (1990). *Annu. Rev. Biochem.* **59**, 331–353.

Rambadran, R. S., and Beattie, D. S. (1992). *Arch. Biochem. Biophys.* **296**, 279–285.

Scherer, P. E., Kreig, U. C., Hwang, S. T., Vestweber, D., and Schatz, G. (1990). *EMBO J.* **9**, 4315–4322.

Sen, K., and Beattie, D. S. (1986). *Arch. Biochem. Biophys.* **251**, 239–249.

Sidhu, A., and Beattie, D. S. (1983). *J. Biol. Chem.* **258**, 10649–10656.

Sidhu, A., Clejan, L., and Beattie, D. S. (1983). *J. Biol. Chem.* **258**, 12308–12314.

Trumpower, B. L., and Gennis, R. B. (1994). *Annu. Rev. Biochem.* **63**, 675–716.