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The role of the membrane-spanning and extra-membranous regions of the iron–sulfur protein in its assembly into the cytochrome bc_1 complex of yeast mitochondria [1]

V. Obungu, L.P. Yu, S. Japa, D.S. Beattie *

Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV 26506-9142, USA

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Abstract

The assembly of six deletion mutants of the Rieske iron–sulfur protein into the cytochrome bc_1 complex was investigated by immunoprecipitation from detergent-solubilized mitochondria with specific antisera against either the iron–sulfur protein or the intact cytochrome bc_1 complex. After import, the mutant proteins lacking residues 41–55 or 66–78, located at the membrane-spanning region of the protein, and residues 182–196 located at the C-terminus of the protein, were assembled in vitro into the bc_1 complex approximately 50% as effectively as the wild type iron–sulfur protein suggesting that these regions of the iron–sulfur protein may not be critical for the assembly. By contrast, only trace amounts of the mutant proteins lacking residues 80–95, 122–135, 138–153 located in the extra-membranous region of the iron–sulfur protein were assembled into the bc_1 complex. After import in vitro into mitochondria isolated from a cytochrome *b*-deficient yeast strain, the mutants lacking residues 41–55 and 182–196 were assembled as efficiently as the wild type; however, the mutants lacking residues 55–66 and 66–78 were assembled less efficiently in the absence of cytochrome *b* suggesting that the hydrophobic membrane-spanning region, residues 55–78, of the iron–sulfur protein, may interact with cytochrome *b* during the assembly of the bc_1 complex. © 1997 Elsevier Science B.V.

Keywords: Cytochrome bc_1 complex; Cytochrome; Mitochondrion; Iron–sulfur protein; Assembly

1. Introduction

The cytochrome bc_1 complex present in the mitochondria of the yeast *Saccharomyces cerevisiae* contains 10 subunits, while that present in bovine heart contains 11 subunits [1]. With the exception of cytochrome *b*, the sole mitochondrial gene product of the bc_1 complex, all the subunits of this complex are synthesized on free cytoplasmic ribosomes and in a subsequent step imported into mitochondria where they are assembled into a functional complex in the inner mitochondrial membrane [2]. The iron–sulfur

Abbreviations: Cytochrome bc_1 complex or complex III, ubiquinol:cytochrome *c* oxidoreductase; Rip or ISP, iron–sulfur protein; RIP, iron–sulfur protein gene; rip, mutant form of iron–sulfur protein gene; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

* Corresponding author. Fax: +1 304 2936846; E-mail: dbeattie@wvuhsc1.hsc.wvu.edu

[1] The amino acid residue numbers used throughout this paper are those of the yeast precursor protein [1]. The residue numbers of the beef mature protein can be obtained by subtracting 20 from the numbers presented here.

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protein, a catalytic subunit of the bc_1 complex, is synthesized as a precursor form with a 30 amino acid bipartite presequence containing positively charged and hydroxylated amino acids at the N-terminus of the protein [3]. This precursor form is processed via an intermediate form into the mature form by 2 proteolytic cleavage steps both in vivo and in vitro in both *S. cerevisiae* and *Neurospora crassa* [4–6]. The mature form of the iron–sulfur protein is anchored on the outer surface of the inner mitochondrial membrane where it protrudes into the inter-membrane space, a localization consistent with its catalytic function in the transfer of electrons from ubiquinol to cytochrome *c* [7,8].

The mechanism of assembly of imported proteins, including the iron–sulfur protein, into functional complexes in 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 occurred at different rates in an ordered manner [4,9]. This experimental approach had indicated that the iron–sulfur protein may be one of the last proteins to become associated with a postulated 'core' membrane-bound complex during mitochondrial biogenesis. In previous studies in our laboratory, we investigated the assembly of the iron–sulfur protein into the bc_1 complex in vitro by using selective immunoprecipitation with antiserum against either the iron–sulfur protein or the intact bc_1 complex after import of radiolabeled protein [10]. More recently, the import and assembly of two deletion mutants of the iron–sulfur protein, one lacking amino acid residues 55–66 and the other lacking residues 161–180, were studied both in vitro and in vivo. The results obtained indicated that the deletion mutant Rip(Δ55–66), 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 suggesting that this hydrophobic stretch of amino acids is not required for the assembly of the iron–sulfur protein into the bc_1 complex; however, the deletion mutant Rip(Δ161–180), lacking amino acid residues 161–180, was not assembled into the bc_1 complex [11]. We interpreted these results to suggest that the interaction of the iron–sulfur protein with other subunits of the bc_1 complex in mitochondria may occur through protein–protein interactions occurring outside the inner membrane where amino acid residues 161–180 are located (Fig. 1).

To obtain further evidence in support of the suggestion that the iron–sulfur protein interacts with other subunits of the bc_1 complex through protein–protein interactions occurring outside the inner membrane and not inside the membrane, 6 additional deletion mutants of RIP, the gene for the iron–sulfur protein, were constructed for further studies of the assembly process in vitro. The amino acid residues chosen for deletion include residues 41–55 and 66–78, located in the putative membrane-spanning region, residues 80–95, 122–135, 138–153 and 182–196, located in the extra-membranous region of the protein (Fig. 1). The results of immunoprecipitation studies with the antiserum against complex III that can only immunoprecipitate the iron–sulfur protein when it is associated with other proteins of the bc_1 complex has provided evidence for the suggestion that the amino acid residues in the membranous

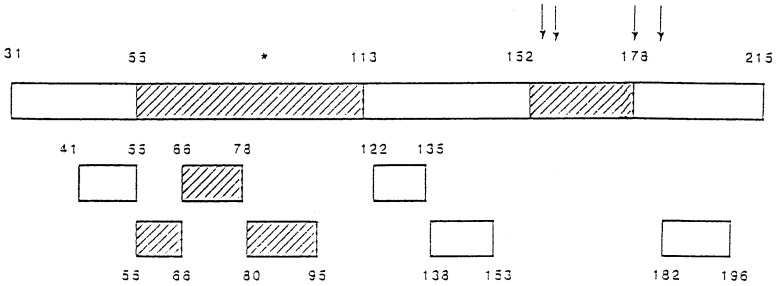

Fig. 1. Model of the iron–sulfur protein indicating the deletion mutants used in this study. The upper bar shows the mature form of the yeast iron–sulfur protein numbered for the precursor form. The open areas in the bar represent hydrophilic regions of the protein and the shaded areas hydrophobic regions [1]. The arrows above the bar indicate the sites of binding of the iron sulfur cluster [19]. Thermolysin cleaves the complete beef heart iron–sulfur protein at residue 87 (indicated by the asterisk) located at the end of the predicted membrane anchor domain [20]. The boxes below the bar indicate the site of the deletion mutants used in this study.

domain as well as the C-terminus of the iron–sulfur protein are *not* required for the assembly of this protein into the bc_1 complex.

2. Materials and methods

2.1. Construction of deletion mutants in the expression vector pSP64-RIP

The expression vector, pSP64-RIP, that contains the gene encoding the iron–sulfur protein was previously constructed in our laboratory [6]. Segments of the RIP gene encoding amino-acid residues 41–55, 66–78, 80–95, 122–135, 138–153 and 182–196 were deleted in this vector using PCR. Anti- and cis-primers containing 26–29 nucleotides were constructed based on the DNA sequences at the 5′- and the 3′- ends of the segments of DNA to be deleted with a *Bam*H1 site introduced at the 5′-end of the primers. The DNA fragments after amplification by PCR were purified and digested with *Bam*HI endonuclease to create cohesive *Bam*H1 sites at both ends. The sticky ends, thus formed, were ligated by T4 DNA ligase in the presence of ATP yielding the mutant constructs pSP64-rip(Δ41–55), rip(Δ66–78), rip(Δ80–95), rip(Δ122–135), rip(Δ138–153) and rip(Δ182–196). The identity of the deletion constructs was confirmed by restriction endonuclease digestion and by DNA sequencing using the dideoxy chain termination sequence method [12].

2.2. Isolation of mitochondria and import in vitro of the wild type and deletion mutants into isolated mitochondria

The yeast strain from which the gene for the iron–sulfur protein had been deleted, JPJ1, [13] and the cytochrome *b*-deficient strain, W-267, were grown aerobically at 30°C in a semi-synthetic medium as described previously [6]. Mitochondria were prepared and suspended in 0.6 M sorbitol containing 20 mM Hepes-KOH, pH 7.4 [6]. The wild-type and the mutant plasmids were transcribed and translated using an in vitro transcription/translation kit supplied by Promega in the presence of [^{35}S]methionine. The reaction mixture was incubated for 2 h at 30°C and then centrifuged at $105\,000 \times g$ for 45 min to remove the ribosomes. The import in vitro of radiolabeled protein into mitochondria from the JPJ1 and W267 cells was performed as previously described [10,11].

2.3. Immunoprecipitation

After the import reaction, the mitochondria were reisolated by centrifugation and the pellet solubilized with 1% Triton X-100 in Buffer A consisting of 0.1 M Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride [10]. Antisera against either the iron–sulfur protein or the intact complex III were added to the solubilized pellets and allowed to incubate overnight at 4°C. After centrifugation at $10\,000 \times g$ for 5 min, Sepharose bound protein A was added and used to collect the immunoprecipitates prior to SDS-PAGE. The reticulocyte lysate after translation was also solubilized in the same buffer containing 1% Triton X-100 prior to incubation with the antisera against the iron–sulfur protein and the intact cytochrome bc_1 complex.

2.4. SDS-PAGE and miscellaneous methods

The samples containing mitochondria after the import reaction and the immunoprecipitates obtained by reactions with the respective antisera were separated by SDS-PAGE [11]. The gels were exposed to a Phosphorimager screen that was then scanned with the PhosphorImager™ and quantitated with the Image QuaNT™ software (Molecular Dynamics, Sunnyvale, CA). DNA isolation and gel electrophoresis were performed by standard techniques [12].

2.5. Materials

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.

3. Results

Import and processing of the mutant iron–sulfur protein precursors into mitochondria from the JPJ1

strain in vitro. After coupled transcription/translation in vitro in the rabbit reticulocyte lysate, radiolabeled precursors of the deletion mutants migrated on SDS-PAGE with apparent molecular weights of 29 000 for the wild type, 27 500 for Rip(Δ44–55), 27 400 for Rip(Δ66–78), 27 400 for Rip(Δ80–97), 26 000 for Rip(Δ122–135), 27 100 for Rip(Δ138–153) and 27 400 for Rip (Δ182–196), as predicted from the gene sequence (Fig. 2A and B, lanes 1, 3, 5, and 7). All of the deletion mutants were imported in vitro into mitochondria of the strain JPJ1, lacking the

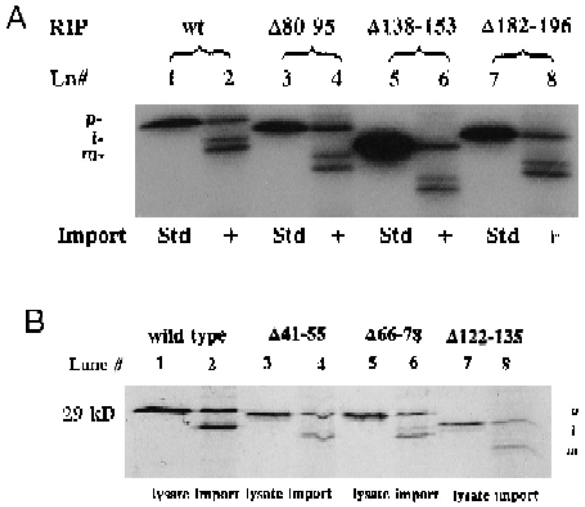

Fig. 2. Import in vitro of the deletion mutants Rip(Δ41–55), Rip(Δ66–78), Rip(Δ80–95), Rip(Δ122–135), Rip(Δ138–153) and Rip(Δ182–196) into yeast mitochondria. The precursor forms of the wild type and mutant iron–sulfur proteins were translated in the presence of [^{35}S]methionine in a coupled in vitro transcription/translation system. The import buffer containing 0.6 M sorbitol/20 mM Hepes/KOH, 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 was preincubated with mitochondria (200 μg of protein) in a final volume of 200 μl for 5 min at 27°C. Then, 10 μl of centrifuged reticulocyte lysate were added to the import mixture and incubated for 30 min at 27°C. Mitochondria were re-isolated from the import mixture by centrifugation at 10 800 × *g* for 5 min in a refrigerated microcentrifuge and washed twice with 0.6 M sorbitol/20 mM Hepes/KOH, pH 7.4, prior to SDS-PAGE and fluorography. *A.* Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ80–95); lanes 5 and 6, Rip(Δ138–153); lanes7 and 8 Rip(Δ182–196). Std; precursor lysate at 10% of the volume used in the import studies. *B.* Lanes 1 and 2, wild type iron–sulfur protein, lanes 3 and 4, Rip(Δ41–55); lanes 5 and 6, Rip(Δ66–78); lanes 7 and 8, Rip(Δ122–135). The positions of precursor (p), intermediate (i) and mature (m) forms of the iron–sulfur protein are indicated. Each experiment was repeated 3 times.

iron–sulfur protein, when incubated in the presence of an ATP-generating system and respiratory substrates including ascorbate and TMPD which are oxidized by cytochrome *c* oxidase thus by-passing the bc_1 complex (Fig. 2A and B). After import, all of the mutants underwent the identical two-step cleavage process as the wild type iron–sulfur protein to the intermediate and then the mature forms. Both the intermediate and mature forms after import were resistant to digestion by exogenous proteinase K suggesting that these forms had been translocated into a protease-resistant compartment of the mitochondria, while the precursor forms were completely digested by proteinase K suggesting that they are located outside the mitochondrial membranes (data not shown). Strain JPJ1, that lacks the iron–sulfur protein, was chosen for studies of the assembly of the bc_1 complex, as we noted in a previous study that significantly more of the iron–sulfur protein was assembled into the bc_1 complex in mitochondria from strain JPJ1 than from the wild type.

3.1. Assembly of the deletion mutants, Rip(Δ41–55), Rip(Δ66–78), Rip(Δ80–95), Rip(Δ122–135, Rip(Δ138–153), and Rip(Δ182–196) into the cytochrome bc_1 complex in vitro

The assembly of the mature form of the iron–sulfur protein into the bc_1 complex after import in vitro has been studied in our laboratory using specific antisera against the iron–sulfur protein and the intact bc_1 complex [10,11]. The labeled precursor form of the wild type iron–sulfur protein was immunoprecipitated from the reticulocyte lysate after translation in vitro with the antiserum against the iron–sulfur protein; however, the antiserum raised against the intact complex III did not immunoprecipitate the precursor form of the iron–sulfur protein (Fig. 3A and B, lanes 1 and 2). Previous studies had indicated that this antiserum raised against complex III *did not recognize the iron–sulfur protein*, but did recognize core protein I, core protein II, cytochrome *b*, cytochrome c_1 and subunits 6 and 7 in immunoblots of the bc_1 complex [14].

Before comparing the assembly of these mutant proteins into the bc_1 complex, it was necessary to establish that the antiserum against the iron–sulfur protein could recognize these proteins in which se-

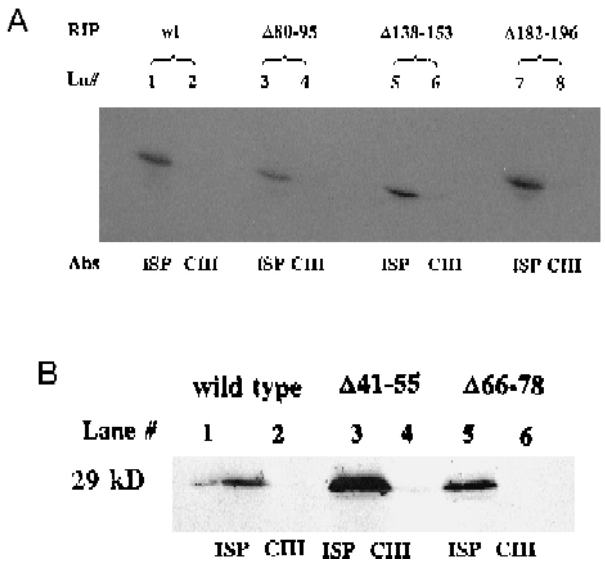

Fig. 3. Immunoprecipitation of the precursor forms of the mutant iron–sulfur proteins by antisera against the intact cytochrome bc_1 complex and the iron–sulfur protein. The translation mixture containing the [^{35}S]methionine-labeled precursor forms of the mutant constructs was added to a mixture containing 0.1 M Tris-HCl, pH 7.4,150 mM NaCl, 2 mM EDTA, 1 mM PMSF and 1% Triton X-100 and incubated with the antisera against the intact bc_1 complex (CIII) and the iron–sulfur protein (ISP), respectively. The immunoprecipitates were subjected to SDS-PAGE and analyzed by fluorography. *A*. Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ80–95); lanes 5 and 6, Rip(Δ138–153); lanes 7 and 8, Rip(Δ182–196). *B*. Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ41–55); lanes 5 and 6, Rip(Δ66–78).

lected regions had been deleted. The labeled precursor forms of all of the deletion mutants of the iron–sulfur protein were immunoprecipitated from the reticulocyte lysate, solubilized with 1% Triton X-100 in Buffer A, by the antiserum against the iron–sulfur protein indicating that this antiserum did indeed recognize these proteins; however, the complex III antiserum did not recognize these labeled precursor forms in the reticulocyte lysate confirming our previous results that the antiserum against the bc_1 complex cannot recognize the iron–sulfur protein (Fig. 3A and B).

The assembly of the deletion mutants into the bc_1 complex was studied in yeast mitochondria solubilized with 1% Triton X-100 after import in vitro and incubated with the antisera against the iron–sulfur protein and against complex III. It should be noted that solubilization of the mitochondrial membranes with 1% Triton X-100 under these conditions did not result in any loss of ubiquinol:cytochrome *c* oxidoreductase activity suggesting that the cytochrome bc_1 complex has remained intact [11]. Addition of antisera against both the iron–sulfur protein and complex III immunoprecipitated the labeled mature form of the wild type iron–sulfur protein suggesting that the radiolabeled protein has been assembled into the bc_1 complex after import in vitro into yeast mitochondria (Fig. 4A and B, lanes 1 and 2).

The efficiency of assembly of the wild type and mutant iron–sulfur proteins into the bc_1 complex was determined by first making the assumption that the amount of radioactivity in the immunoprecipitate formed with the antiserum against the iron–sulfur protein, quantitated by the Phosphorimager, repre-

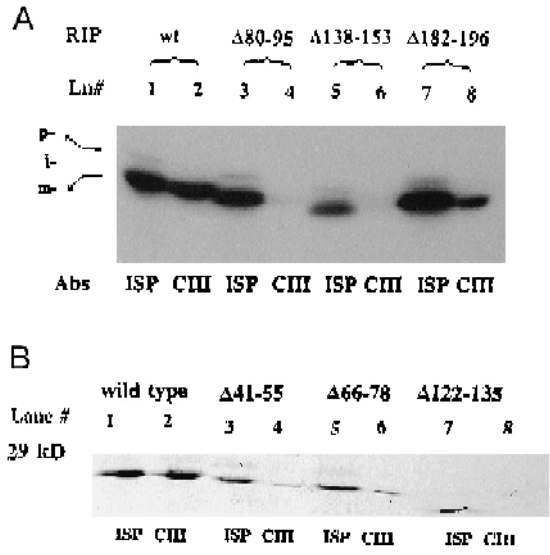

Fig. 4. Assembly of the deletion mutants, Rip(Δ41–55), Rip(Δ66–78) Rip(Δ80–95), Rip(Δ–135), Rip(Δ138–153) and Rip(Δ182–196), into the bc_1 complex after import in vitro into mitochondria of JPJ1 cells. Isolated mitochondria were incubated in the import medium as described in the legend to Fig. 2 and re-isolated prior to solubilization. The re-isolated mitochondria were solubilized with 1% Triton X-100 and incubated with the antisera against the bc_1 complex (CIII) and the iron–sulfur protein (ISP). The immunoprecipitates were subjected to SDS-PAGE and then analyzed by fluorography. (A) Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ80–95); lanes 5 and 6, Rip(Δ138–153); lanes 7 and 8, Rip(Δ182–196). (B) Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ41–55); lanes 5 and 6, Rip(Δ66–78); lanes 7 and 8, Rip(Δ122–135). The positions of the precursor (p), intermediate (i) and mature (m) forms of the iron–sulfur protein are indicated.

sents the total imported iron–sulfur protein. The percentage of assembly was then estimated by dividing the radioactivity in the immunoprecipitate obtained with the antibody against the bc_1 complex (complex III) from mitochondria solubilized with Triton X-100 by the radioactivity immunoprecipitated with the antibody against the iron–sulfur protein. Using this calculation, we estimated that 72–95% of the mature wild type iron–sulfur protein imported into mitochondria was assembled into the bc_1 complex (Table 1), a result comparable to previous studies in our laboratory [10,11].

Table 1 also indicates that varying amounts of the deletion mutants were immunoprecipitated with the antiserum against complex III reflecting the different efficiencies of assembly of these mutant iron–sulfur proteins into the bc_1 complex (Fig. 4A and B, lanes 2, 4, 6 and 8). To compare the assembly of the mutant proteins into the bc_1 complex to that of the wild type iron–sulfur protein, the efficiency of assembly of the wild type was set to 100% and that of the mutants calculated as a percentage of their assembly compared to the wild type. The efficiencies of assembly of the deletion mutants varied with the location of the deleted amino acids in the iron–sulfur protein (Fig. 1). Two of the mutants containing deletions in the putative membrane-spanning regions of the iron–sulfur protein, Rip(Δ41–55) and Rip(Δ66–78) were assembled nearly 50% as well as the wild type protein as was the mutant containing a deletion at the C-terminus of the protein, Rip(Δ182–196) suggesting that these regions of the protein may not be critical for the assembly of the iron–sulfur protein into the bc_1 complex. By contrast, the other three deletion mutants, Rip(Δ80–95), Rip(Δ122–135) and Rip(Δ138–153), were not efficiently assembled into the bc_1 complex. The possibility that changes in the tertiary structure of the iron–sulfur protein resulting from these deletions may have resulted in improper assembly make it difficult to conclude that these regions are required for assembly.

Table 1
Assembly of deletion mutants into the bc_1 complex after import into mitochondria of yeast strain JPJ1

Iron–sulfur protein	% Assembly	Relative efficiency to wild type [a]
Expt. 1		
Wild type Rip	72	100
Rip(Δ80–95)	6	9
Rip(Δ138–153)	4	12
Rip(Δ182–196)	33	46
Expt. 2		
Wild type Rip	95	100
Rip(Δ41–55)	41	43
Rip(Δ66–78)	38	40
Rip(Δ122–136)	12	13

[a] The percent efficiency of assembly of the wild type iron–sulfur protein (Rip) was set at 100%.
The percent assembly was calculated by dividing the radioactivity in the iron–sulfur protein immunoprecipitated by the complex III antiserum by the radioactivity in the iron–sulfur protein immunoprecipitated by the iron–sulfur protein antiserum after import in vitro into mitochondria isolated from JPJ1, the yeast strain from which the RIP gene was deleted. See legend to Fig. 4 for experimental details. Each value is the average of 3 different experiments.

3.2. Assembly of the deletion mutants Rip(Δ41–55), Rip(Δ55–66), Rip(Δ66–78), and Rip(Δ182–196) into yeast mitochondria lacking cytochrome b

Cytochrome *b*, the most hydrophobic subunit of the bc_1 complex, spans the inner mitochondrial membrane in eight transmembrane α helices [1,14]. Previous experiments had indicated that cytochromes *b* and c_1 are the first polypeptides to be assembled into the complex, where they may function as a template onto which other subunits of the complex are then assembled [4,9]. To test this suggestion and to examine the role of cytochrome *b* in the assembly of the bc_1 complex we examined the import and assembly in vitro of the wild type iron–sulfur protein and several deletion mutants into mitochondria lacking cytochrome *b*. The absence of cytochrome *b* results in the loss of many of the smaller subunits of the bc_1 complex; however, the levels of cytochrome c_1 and the two core proteins are at or near wild type levels in this strain [15].

The mutants chosen for this study, Rip(Δ41–55), Rip(Δ55–66) (11), Rip(Δ66–78) and Rip(Δ182–196), were assembled with an efficiency approaching 50% into the bc_1 complex of mitochondria isolated from the yeast strain, JPJ1, from which the RIP gene had been deleted [13]. All 4 deletion mutants were imported into the mitochondria lacking cytochrome *b*

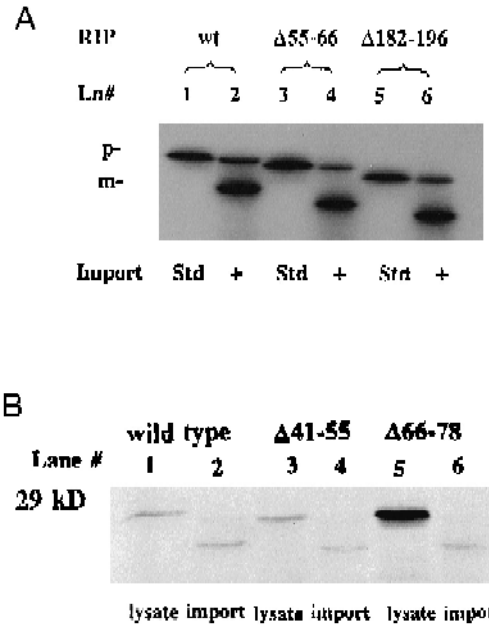

Fig. 5. Import in vitro of the deletion mutants Rip(Δ41–55), Rip(Δ55–66), Rip(Δ66–78) and Rip(Δ182–196) into cytochrome *b*-deficient mitochondria. Mitochondria were incubated in the import medium as described in the legend to Fig. 1 and subjected to SDS-PAGE. (A) Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ55–66); lanes 5 and 6, Rip(Δ182–196). (B) Lanes 1 and 2, wild type; lanes 3 and 4 Rip(Δ41–55); lanes 5 and 6, Rip(Δ66–78). Std; precursor lysate at 10% of the volume used in the import studies. The positions of precursor (p), and mature (m) forms of the iron–sulfur proteins are indicated.

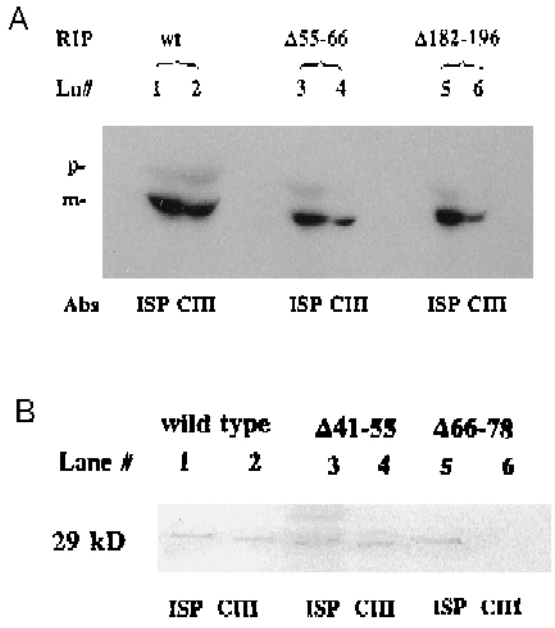

Fig. 6. Assembly of the deletion mutants Rip(Δ41–55), Rip(Δ55–66) and Rip(Δ182–196) into the bc_1 complex after import in vitro into cytochrome *b*-deficient mitochondria. Mitochondria were incubated in the import medium as described in the legend to Fig. 1. After import the mitochondria were solubilized with 1% Triton X-100 and incubated with the antisera against the bc_1 complex (CIII) and the iron–sulfur protein (ISP) prior to SDS-PAGE and fluorography. (A) Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ55–66); lanes 5 and 6, Rip(Δ182–196). (B) Lanes 1 and 2, wild type; lanes 3 and 4, Rip(Δ41–55); and lanes 5 and 6, Rip(Δ66–78). The positions of precursor (p), and mature (m) forms of the iron–sulfur protein are indicated.

Table 2
Assembly of deletion mutants into the bc_1 complex after import into mitochondria lacking cytochrome *b*

Iron–sulfur protein	Assembly	Relative efficiency to JPJI [a]
Expt. 1		
Wild type Rip	50	71
Rip(Δ55–66)	39	56
Rip(Δ182–196)	37	112
Expt. 2		
Wild type Rip	81	85
Rip(Δ41–55)	46	112
Rip(Δ66–78)	17	45

[a] The percent assembly into W267 was divided by the percent assembly into JPJ1 to obtain the relative efficiency.
The percent assembly was calculated as described in the legend to Table 1 after import into mitochondria isolated from the yeast strains W267, lacking cytochrome *b*. See legend to Fig. 6 for experimental details. Each value is the average of 3 separate experiments.

and processed to the mature form as efficiently as the wild type iron–sulfur protein (Fig. 5A and B). The efficiency of assembly of the wild type iron–sulfur protein into the bc_1 complex of the b-deficient mitochondria varied from 50–81%, a value significantly lower than that observed with the mitochondria of JPJ1 suggesting that cytochrome *b* is required for optimal assembly. The efficiency of assembly of the deletion mutants into the bc_1 complex of the b-deficient mitochondria was 46% for Rip(Δ41–55), 39% for Rip(Δ55–66), 17% for Rip(Δ66–78) and 37% for Rip(Δ182–196) (Fig. 6A and B; Table 2). Comparing the efficiency of assembly of these mutants into the cytochrome *b*-deficient compared to the iron–sulfur protein deficient mitochondria indicates that both Rip(Δ41–55) and Rip(Δ182–196) were

assembled with equal or better efficiencies into the cytochrome *b*-deficient mitochondria suggesting that these amino acids do not interact with cytochrome *b* during the assembly process. By contrast, the deletion mutants Rip(Δ55–66) and Rip(Δ66–78) were assembled only 50% as efficiently into the cytochrome *b*-deficient compared to the JPJ1 mitochondria suggesting that these amino acids may interact with cytochrome *b* for optimal assembly of the iron–sulfur protein into the bc_1 complex.

4. Discussion

In the current study, 6 deletion mutants of the iron–sulfur protein have been used to obtain additional information as to which regions of this protein may be involved in its assembly in vitro into the bc_1 complex of yeast mitochondria. Previously, the assembly of 2 deletion mutants, one lacking amino acid residues 55–66, located in a hydrophobic and proposed membrane-spanning region of the protein, and one lacking residues 161–181, located in the extra-membranous region of the protein surrounding the FeS clusters was investigated. The results obtained in that study suggested that during assembly of the bc_1 complex the iron–sulfur protein may interact with other subunits of the complex through hydrophilic/hydrophobic interactions of regions of the protein localized outside the membrane rather than through protein/protein interactions of the putative hydrophobic membrane-spanning domain [11]. To obtain additional experimental evidence in support of this suggestion, the import and assembly of 4 deletion mutants localized in the extra-membranous region and 2 deletion mutants localized in the putative membrane-spanning regions of the iron–sulfur protein were investigated.

Two of the deletion mutants studied, one lacking amino acid residues 41–55 and the other amino acid residues 66–78, were imported into mitochondria and processed to the mature form which was assembled into the bc_1 complex with an efficiency nearly 50% that of the mature iron–sulfur protein. These results suggest that the amino acid residues deleted in these mutants may not be critical for the interaction of the iron–sulfur protein with other subunits during the assembly of the bc_1 complex. The amino acid residues deleted in these 2 mutants are localized in or near a single α-helical transmembrane domain proposed to anchor the iron–sulfur protein to the membrane [16]. Moreover, these deleted amino acid residues flank the amino acid residues, 55–66, which our previous results suggested were not involved in the assembly of the iron–sulfur protein into the bc_1 complex both in vitro and in vivo [11].

By contrast, 3 mutants containing deletions in amino acid residues, 80–95, 122–135, and 138–153, all localized in the extra-membranous region of the iron–sulfur protein, were not assembled appreciably into the bc_1 complex. The lack of assembly may have resulted because these deletions resulted in improper folding of the iron–sulfur protein such that it cannot assemble with other proteins of the bc_1 complex. By contrast, the mutant lacking residues 182–196 localized at the C-terminus of the protein was assembled into the bc_1 complex with an efficiency 50% that of the wild type protein suggesting that this extra-membranous region of the protein may not be critical for the assembly of the iron–sulfur protein into the bc_1 complex. The decreased efficiency of assembly in this mutant suggests that deleting these amino acids may change the conformation of the protein thus preventing its complete assembly into the complex. The results obtained with all 6 deletion mutants provide additional evidence to suggest that the interaction of the iron–sulfur protein with other subunits of the bc_1 complex occurs largely by hydrophilic/hydrophobic interactions of the extra-membranous domain of the protein.

To determine the role of cytochrome *b* in the assembly of the iron–sulfur protein into the bc_1 complex we used mitochondria from a cytochrome *b*-deficient strain of yeast for import and assembly studies in vitro [17]. The wild type iron–sulfur protein was assembled less efficiently into these mitochondria compared to those isolated from yeast strain JPJ1, an indication that cytochrome *b* does play a role in the assembly in vitro of the iron–sulfur protein into the bc_1 complex. Comparing the efficiency of assembly of 4 deletion mutants into the bc_1 complex of mitochondria lacking cytochrome *b* with that observed with the mitochondria from JPJ1 provided a preliminary suggestion as to the regions of the iron–sulfur protein which may interact with cytochrome *b*, for example, 2 of the mutants, those lacking amino

acid residues 55–66 or residues 66–78 were significantly less efficiently assembled than the wild type protein into the cytochrome *b*-deficient mitochondria suggesting that the putative trans-membranous domain of the iron–sulfur protein may interact with cytochrome *b* during the association of the iron–sulfur protein with the other subunits of the bc_1 complex. The absence of these amino acids in the iron–sulfur protein coupled with the absence of cytochrome *b* may prevent the optimal assembly of these deletion mutants into the bc_1 complex. By contrast, 2 mutants, those lacking amino acid residues 41–55 and residues182–196, were assembled equally well into the cytochrome *b*-deficient mitochondria as into the mitochondria lacking the iron–sulfur protein suggesting that cytochrome *b* may not interact with these regions of the iron–sulfur protein during the assembly of the bc_1 complex. Instead, these regions of the iron–sulfur proteins may interact with either cytochrome c_1 or the core proteins which are present at or near wild type levels in mitochondria of the cytochrome *b*-deficient strain [15].

A model for the assembly of the cytochrome bc_1 complex consistent with these results suggests that the iron–sulfur protein may be integrated into the bc_1 complex of mitochondria through multiple interactions of different regions of the protein with other subunits of the bc_1 complex by a process involving both hydrophobic and hydrophilic interactions. To obtain more precise information about the interactions of the iron–sulfur protein with other subunits during the assembly process site-directed mutagenesis of amino acid residues in the hydrophilic extramembranous regions of the iron–sulfur protein is currently underway in our laboratory. Our preliminary data indicate that a patch of several charged amino acid residues, D-145, R-146, and D-149, located in a loop connecting beta strands in the iron–sulfur protein [18] may be involved in the assembly of the iron–sulfur protein into the cytochrome bc_1 complex.

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