

## Intermediate Length Rieske Iron-Sulfur Protein Is Present and Functionally Active in the Cytochrome $bc_1$ Complex of *Saccharomyces cerevisiae*\*

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To investigate the relationship between post-translational processing of the Rieske iron-sulfur protein of *Saccharomyces cerevisiae* and its assembly into the mitochondrial cytochrome  $bc_1$  complex we used iron-sulfur proteins in which the presequences had been changed by site-directed mutagenesis of the cloned iron-sulfur protein gene, so that the recognition sites for the matrix processing peptidase or the mitochondrial intermediate peptidase (MIP) had been destroyed. When yeast strain JPJ1, in which the gene for the iron-sulfur protein is deleted, was transformed with these constructs on a single copy expression vector, mitochondrial membranes and  $bc_1$  complexes isolated from these strains accumulated intermediate length iron-sulfur proteins *in vivo*. The cytochrome  $bc_1$  complex activities of these membranes and  $bc_1$  complexes indicate that intermediate iron-sulfur protein (i-ISP) has full activity when compared with that of mature sized iron-sulfur protein (m-ISP). Therefore the iron-sulfur cluster must have been inserted before processing of i-ISP to m-ISP by MIP. When iron-sulfur protein is imported into mitochondria *in vitro*, i-ISP interacts with components of the  $bc_1$  complex before it is processed to m-ISP. These results establish that the iron-sulfur cluster is inserted into the apoprotein before MIP cleaves off the second part of the presequence and that this second processing step takes place after i-ISP has been assembled into the  $bc_1$  complex.

The Rieske iron-sulfur protein is an essential subunit of mitochondrial cytochrome  $bc_1$  complexes (1). Like the majority of mitochondrial proteins it is encoded by a nuclear gene, translated on cytosolic ribosomes, and then targeted to the mitochondria by an amino-terminal presequence (2, 3). During import and assembly of the iron-sulfur protein into *Saccharomyces cerevisiae* mitochondria (4–6), a 30-amino acid amino-terminal targeting sequence is removed in two steps. A matrix processing peptidase (MPP)<sup>1</sup> first removes a 22-amino acid peptide from the presequence of the precursor iron-sulfur pro-

tein (p-ISP) to form intermediate iron-sulfur protein (i-ISP). Because MPP is a matrix protein (7–10), this cleavage step presumably takes place in the mitochondrial matrix. A mitochondrial intermediate peptidase (MIP) then removes an octapeptide from i-ISP to generate mature length iron-sulfur protein (m-ISP). It is not clear, however, whether this last processing step takes place before or after the protein is inserted into the  $bc_1$  complex. It is also not known at what step during proteolytic processing the 2 Fe:2 S cluster is inserted into the apoprotein.

Although previous experiments indicated that i-ISP can be found in the  $bc_1$  complex *in vivo* (11–13), *in vitro* studies suggested that only m-ISP can be assembled into the complex (5). In the present study we employed yeast strains that express iron-sulfur proteins in which the recognition sites for MPP or MIP in the presequence have been destroyed by site-directed mutagenesis. Mitochondrial membranes isolated from these strains contain only minor amounts of mature length iron-sulfur protein, but their cytochrome  $c$  reductase activities are comparable with membranes from wild type yeast, indicating that the iron-sulfur cluster is inserted into the protein before MIP processes i-ISP to m-ISP. Immunoprecipitation of  $bc_1$  complex after import of iron-sulfur protein into mitochondria *in vitro* demonstrates that intermediate iron-sulfur protein is assembled into the  $bc_1$  complex.

### EXPERIMENTAL PROCEDURES

**Materials**—Reagents for *in vitro* transcription and translation of proteins were from Promega. The *in vitro* translation product was labeled using Tran<sup>35</sup>S-label (methionine) from ICN. EDTA was from Fisher, and *o*-phenanthroline was from Sigma. Automated sequencing was performed using the Dye Terminator Sequencing kit from Applied Biosystems Inc. The yeast strains and plasmids used are shown in Table I.

**Isolation of Mitochondria**—*S. cerevisiae* strain W303-1A was grown in 2% yeast extract, 2% peptone, 2% dextrose at 30 °C to an optical density at 600 nm of 2–4. Mitochondria were isolated from spheroplasts and frozen as described previously (14).

**In Vitro Transcription and Translation**—*In vitro* transcription and translation using the TnT®-coupled reticulocyte lysate system were performed according to supplier recommendations. Before use in the import experiment, polyribosomes were removed by centrifugation at 230,000 × *g* for 20 min.

**Import of Iron-Sulfur Protein into Mitochondria in Vitro**—The *in vitro* import mixture contained 4–12% (v/v) translated iron-sulfur protein precursor in rabbit reticulocyte lysate and an additional 10–18% (v/v) of rabbit reticulocyte lysate. The mixture also contained 154 mM sucrose, 49 mM KCl, 7 mM MOPS-KOH, pH 7.2, 2.1% bovine serum albumin, 1.4 mM MgCl<sub>2</sub>, 1 mM ATP, 4 mM NADH, and 20–50 μg of mitochondrial protein in a total volume of 0.1 ml. Import was performed as described previously (15).

**Immunoprecipitation**—The immunoprecipitation was performed as described (5) with minor modifications. After import of the labeled iron-sulfur protein into mitochondria *in vitro*, the washed mitochondria from 0.2 ml of import mixture were solubilized with 3% SDS, 1% Triton X-100, or 0.8 mg of dodecyl maltoside/mg of protein in 100 μl of a buffer

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<sup>1</sup> The abbreviations used are: MPP, matrix processing peptidase; MIP, mitochondrial intermediate peptidase; p-ISP, precursor iron-sulfur protein; i-ISP, intermediate iron-sulfur protein; m-ISP, mature iron-sulfur protein; MOPS, 3-(*N*-morpholino) propanesulfonic acid; CAPS, cyclohexylaminopropane sulfonic acid; PAGE, polyacrylamide gel electrophoresis.

TABLE I  
Yeast strains and plasmids

Yeast strain	Transcription plasmid	Expression plasmid	Iron-sulfur protein
JN24	pGem3-RIP	pFL39-RIP	Wild type
JN20	pJN49	pJN63	MIP blocked
JN16	pJN32	pJN38	MPP blocked
CHS14		pFL39:RIP-S183C	S183C

containing 0.1 M Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 5 mM diisopropylfluorophosphate. The SDS-dissociated sample was vigorously shaken at room temperature for 30 min, whereas the samples treated with Triton X-100 or dodecyl maltoside were shaken at 4 °C for 30 min. Subsequently all the samples were diluted 10× with the above buffer containing 1% Triton X-100, and in some experiments 10 mM EDTA and 4 mM *o*-phenanthroline were also included.

For immunoprecipitation the samples were then incubated with the indicated antiserum for 16 h at 4 °C. After centrifugation at 16,000 × *g* for 3 min 100 μl of agarose bound protein G (Boehringer Mannheim) was added to the supernatants, and the mixture was incubated with shaking for 1 h at room temperature. The agarose beads were collected by centrifugation and then washed two times for 20 min at 4 °C with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.2% Triton X-100 and two times in the same buffer without Triton X-100. The washed agarose-bound precipitates were resuspended in 50 μl of 2× concentrated SDS-PAGE sample buffer and incubated at 95 °C for 5 min. The beads were removed by centrifugation at 16,000 × *g* for 1 min, and the supernatants were analyzed by SDS-PAGE and fluorography of the dried gels.

**Amino-terminal Sequencing**—Purified *bc*<sub>1</sub> complex from strain JN16 was subjected to SDS-PAGE and then transferred to Pro Blott™ membranes using 10 mM CAPS, pH 11, 10% methanol as transfer buffer. After drying between blotting paper the bands of interest were excised and subjected to NH<sub>2</sub>-terminal sequencing in an Applied Biosystems Protein Sequencer (model 476A).

**Western Analysis of Mitochondrial Membranes and *bc*<sub>1</sub> Complexes**—Mitochondrial membranes (15) or purified *bc*<sub>1</sub> complexes (16) were resolved on 15% SDS-PAGE gels (17) and either stained with Coomassie blue or blotted to nitrocellulose membranes. Iron-sulfur protein and cytochrome *c*<sub>1</sub> were detected by Western blotting (18) using monoclonal antibodies to the iron-sulfur protein or cytochrome *c*<sub>1</sub>. The intensities of the signals were quantified using an Apple Color One Scanner and the NIH Image software.

**Cytochrome *c* Reductase Activity Measurements**—Ubiquinol-cytochrome *c* oxidoreductase activities of mitochondrial membranes and purified *bc*<sub>1</sub> complexes were assayed in 50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 0.2 mM EDTA, 1 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.1% (w/v) bovine serum albumin at 23 °C using 80 μM 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinol as substrate and 50 μM cytochrome *c*. Reduction of cytochrome *c* was monitored in an Aminco DW-2a™ spectrophotometer at 550 versus 539 nm in dual wavelength mode. Data were collected and analyzed using an Online Instrument Systems Inc. computer interface and software. Turnover numbers of the *bc*<sub>1</sub> complex in membranes and of the purified enzymes were calculated on the basis of the concentration of cytochrome *b*, which was determined from optical spectra of the dithionite reduced minus ferricyanide oxidized samples (19). For each yeast strain membranes and purified enzymes from three individual isolations were assayed in triplicate. Activities are expressed as a percentage of the turnover number of membranes or *bc*<sub>1</sub> complex from yeast strain JN24, carrying wild type RIP1 on a low copy expression vector. The turnover numbers of the enzyme from JN24 ranged between 200 and 220 s<sup>-1</sup>, as reported previously for the enzyme from wild type strains (20).

**Site-directed Mutagenesis of the Iron-Sulfur Protein Gene**—Site-directed mutagenesis was performed using the CLONTECH Transformer Mutagenesis kit. The plasmid pGem3-RIP, carrying the RIP1 gene for the iron-sulfur protein, was used as the template for construction of pJN49. For construction of pJN38 and pJN63 the *Hind*III-*Pst*I fragment of YEP351-RIP1, encoding iron-sulfur protein and the promoter region, was subcloned into pGem3, and the resulting vector was used as template for site-directed mutagenesis. The mutagenized fragments were then excised with *Hind*III and *Pst*I and subcloned into the expression vector pFL39:RIP1, a CEN, TRP vector carrying the gene encoding iron-sulfur protein on a *Hind*III-*Sac*I fragment. The construction of pFL39:RIP-S183C has been previously described (21).

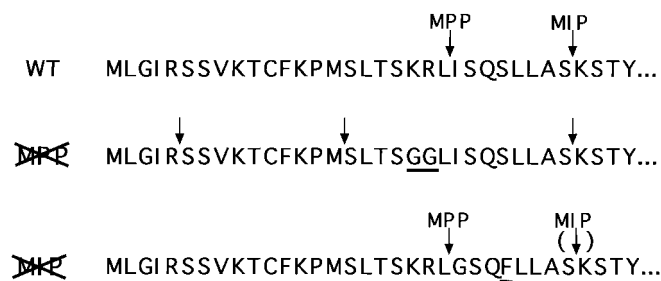


FIG. 1. Amino acid sequences of the mutagenized presequences used in this study. Shown are the presequence of the Rieske iron-sulfur protein and the amino acid replacements that were made by site-directed mutagenesis of the cloned gene. Amino acids that were introduced by site-directed mutagenesis are *underlined*. The MPP and MIP processing sites are marked by solid arrows (↓). The arrow in parentheses indicates that MIP cuts at this site with very low activity. It has not been determined which proteases are responsible for the processing steps in JN16, in which the MPP site is blocked. The first two processing sites indicated by arrows have been determined by sequencing of the intermediate length proteins in purified *bc*<sub>1</sub> complex isolated from this strain.

## RESULTS

**Intermediate Length Iron-Sulfur Protein Is Assembled into the *S. cerevisiae bc*<sub>1</sub> Complex When the MPP or MIP Processing Site in the Presequence Is Destroyed**—In *Neurospora crassa* and *S. cerevisiae*, p-ISP is translocated into the matrix, where it is processed to i-ISP by MPP (4, 22). The remaining octapeptide is subsequently removed by MIP to generate m-ISP. However, it has not been established where this last processing step takes place. We previously reported that i-ISP can be found in isolated *bc*<sub>1</sub> complex (11–13), suggesting that i-ISP is assembled into the *bc*<sub>1</sub> complex and that processing of i-ISP to m-ISP occurs in the *bc*<sub>1</sub> complex. On the other hand, the results of other studies had suggested that only mature length iron-sulfur protein can be assembled into the *bc*<sub>1</sub> complex (5).

To test the hypothesis that i-ISP is assembled into the *bc*<sub>1</sub> complex we constructed yeast expression plasmids that generate iron-sulfur proteins in which the MPP or the MIP recognition sites have been destroyed. We have shown previously that changing Lys-20 and Arg-21 to glycines in the yeast iron-sulfur protein presequence results in complete inhibition of processing by MPP and accumulation of precursor iron-sulfur protein *in vitro* (Fig. 1 and Refs. 23 and 24).

The MIP recognition site is defined by a large hydrophobic residue in position -8 and a small hydroxylated residue or glycine in position -5 from the final MIP cleavage site (25), equivalent to residues Ile-23 and Ser-26 in the yeast iron-sulfur protein presequence. To destroy this site we first changed Ile-23 to glycine. This mutation resulted in accumulation of i-ISP after import *in vitro*; however, a significant amount of m-ISP was still formed (results not shown). To further improve the inhibition of processing by MIP we then also changed Ser-26 into phenylalanine. As shown in Fig. 2, this mutated iron-sulfur protein (pJN49) is processed at an extremely low rate by MIP, and only trace amounts of m-ISP are formed.

To determine whether insertion of the iron-sulfur cluster is required before MIP can perform the final processing step, we also included an iron-sulfur protein in which Ser-183 had been changed to cysteine (pFL39:RIP-S183C). We previously showed that this mutation results in the loss of the iron-sulfur cluster but has no effect on the stability of the protein (21). To determine the effect of these mutations on processing of the iron-sulfur protein presequence, we isolated mitochondrial membranes from yeast strains expressing wild type and mutated iron-sulfur proteins and performed Western blots on them. As shown in Fig. 3A, the main species that is formed when the MIP site is blocked is i-ISP, as was expected. In this strain very

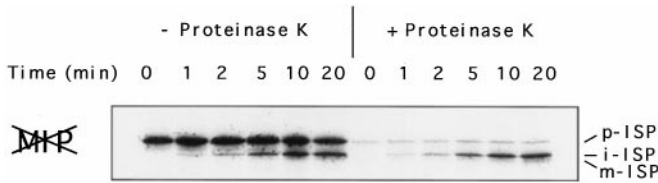


FIG. 2. **Import into mitochondria of iron-sulfur protein containing altered presequence.** Iron-sulfur protein in which the MIP site is blocked was imported into mitochondria *in vitro* for the indicated times. After import half of each sample was treated with proteinase K.

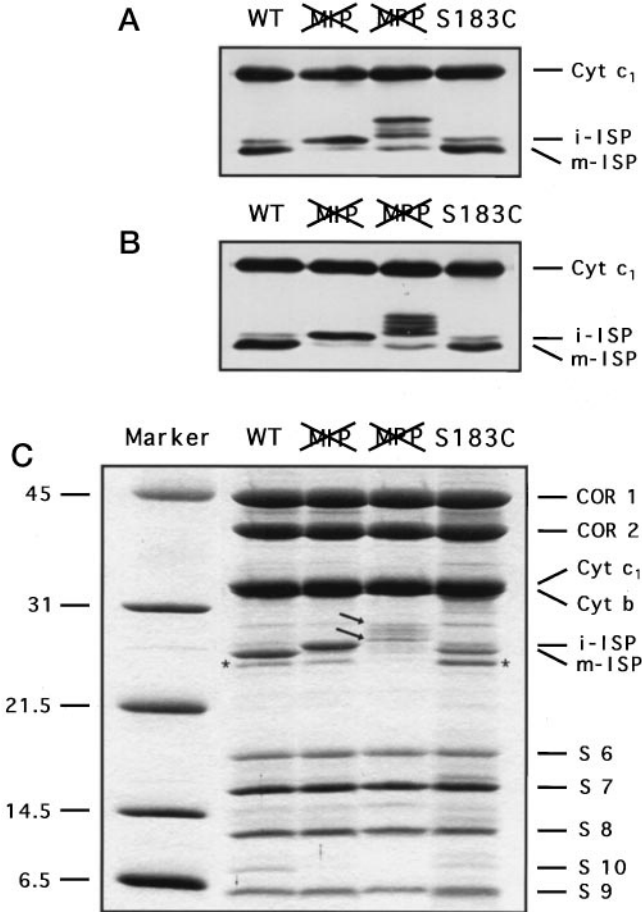


FIG. 3. **Immunoblot analysis and polyacrylamide gel electrophoresis of mutant iron-sulfur proteins in mitochondrial membranes and isolated  $bc_1$  complexes.** Mitochondrial membranes containing 2 pmol of  $bc_1$  complex (A) or 2 pmol of purified  $bc_1$  complexes (B) isolated from yeast strains expressing wild type and mutated iron-sulfur proteins (see Fig. 1) were separated by SDS-PAGE and blotted to nitrocellulose, and the blots were probed with antibodies against iron-sulfur protein and cytochrome  $c_1$ . In C samples containing 110 pmol of cytochrome  $bc_1$  complexes purified from yeast strains expressing wild type or mutated iron-sulfur proteins were separated on a 15% SDS-PAGE gel and stained with Coomassie blue to visualize the protein bands. The arrows point to the two intermediates that were subjected to amino-terminal sequencing. The asterisk marks an impurity that migrates slightly ahead of m-ISP.

little m-ISP is present in the mitochondrial membranes, and the ratio of i-ISP to m-ISP is the inverse of that in mitochondrial membranes from the wild type strain.

In membranes from JN16, where the MPP site has been destroyed, alternative processing of the presequence occurs, such that several intermediates of different lengths can be detected. We determined two of the additional processing sites by  $NH_2$ -terminal sequencing of the intermediates, indicated by arrows in Figs. 1 and 3C. One cleavage occurs after Arg-5 in the presequence, and another one occurs after Met-15 with addi-

tional cleavage sites in between. The basic residues in the presequence are not part of the recognition motif for those cleavage sites, because changing each basic residue in the presequence individually did not alter the processing pattern compared with that in JN16 (results not shown). The iron-sulfur protein that possesses the S183C mutation is processed like the wild type protein, suggesting that insertion of the iron-sulfur cluster is not an obligatory prerequisite for processing (Fig. 3A). However, the relative amount of m-ISP in the S183C form of the iron-sulfur protein is decreased in comparison to that from the wild type strain (Fig. 3C), suggesting that processing from i-ISP to m-ISP is retarded if the cluster is not inserted.

To determine whether the intermediate length forms of the iron-sulfur protein are assembled into the  $bc_1$  complex, we isolated  $bc_1$  complexes from the mutant and wild type strains and performed Western blots on them (Fig. 3B). From the Western blots of the mitochondrial membranes in Fig. 3A and of the purified  $bc_1$  complexes in Fig. 3B, it is clear that basically all of the intermediate length species that are found in the membranes are assembled into the  $bc_1$  complex. In a parallel experiment we stained an SDS-PAGE gel of these  $bc_1$  complexes with Coomassie blue (Fig. 3C). These findings confirm that i-ISP is assembled into the  $bc_1$  complex.

*Intermediate Length Iron-Sulfur Protein Is Functionally Active in the  $bc_1$  Complex of *S. cerevisiae**—To test whether the intermediate length species in the  $bc_1$  complexes of JN16 and JN20 contain iron-sulfur cluster, we determined the cytochrome  $c$  reductase activities of membranes and  $bc_1$  complexes isolated from these strains. We also determined the relative amounts of i-ISP and m-ISP from the intensity of the bands on the Western blots.

From the results in Fig. 4, it is clear that basically all of the i-ISP in the strain in which the MIP site is blocked (JN20) and at least a major portion of the intermediates in the strain in which MPP is blocked (JN16) are functionally active and therefore must contain the iron-sulfur cluster. The densitometry measurements (Fig. 4A) indicate that m-ISP accounts only for about 5–15% of the total iron-sulfur protein when the MIP or MPP processing site is blocked, whereas mitochondrial membranes isolated from these strains exhibit  $bc_1$  complex activity comparable with those from the wild type strain (Fig. 4B).

These results show that i-ISP is assembled and functionally active in the  $bc_1$  complex of *S. cerevisiae*. However, it is noteworthy that the  $bc_1$  complex isolated from JN20, in which the MIP site is blocked, has only about 50% activity when compared with that from the wild type strain. A similar drop in turnover number in the isolated  $bc_1$  complex in comparison to the mitochondrial membranes is observed in the case of JN16. It therefore seems that activity is lost during purification of the complex in which i-ISP is not processed to m-ISP.

*Intermediate Length Iron-Sulfur Protein Can Be Assembled into the  $bc_1$  Complex of *S. cerevisiae* *In Vitro**—To determine whether the intermediate length forms of the iron-sulfur protein also interact with other subunits of the  $bc_1$  complex *in vitro*, we imported wild type iron-sulfur protein and iron-sulfur proteins in which the MIP or MPP recognition sites were destroyed into mitochondria *in vitro*. After import the membranes were extracted with different detergents, and the extracts were precipitated with antibodies against the iron-sulfur protein or antibodies that recognize the  $bc_1$  complex but not the iron-sulfur protein. As shown in Fig. 5A, if membranes are extracted with either Triton X-100 or dodecyl maltoside, which do not denature the  $bc_1$  complex, i-ISP is precipitated by the antibodies against the complex. If the membranes are extracted with SDS, which denatures the  $bc_1$  complex, antibodies raised



into i-ISP while it is still in the matrix or after it has been translocated to the outer side of the inner mitochondrial membrane. The last step of the assembly process then seems to be the processing of i-ISP containing the 2 Fe:2 S cluster to m-ISP by MIP.

In bovine mitochondria the cleaved presequence of the iron-sulfur protein is retained as a subunit in the  $bc_1$  complex (29). In the crystal structure of the bovine  $bc_1$  complex, the amino terminus of m-ISP and the cleaved presequence are located on the matrix side of the complex (30, 31). It thus seems likely that the bovine iron-sulfur protein is processed after it is assembled into the complex (29).

A variation of this mechanism could be applicable to the *S. cerevisiae* iron-sulfur protein. No subunit analogous to the bovine iron-sulfur protein presequence has been identified so far in *S. cerevisiae*. However, in contrast to the bovine iron-sulfur protein, the *S. cerevisiae* presequence is processed in two steps. In unpublished experiments we have found that the 22-amino acid peptide that is removed by MPP is not present in the  $bc_1$  complex or in mitochondrial membranes. However, the octapeptide that is cleaved from i-ISP by MIP may stay in the complex as a yet unidentified subunit. This might explain why mitochondrial membranes isolated from JN20, in which the blocked MIP site prevents formation of the octapeptide, exhibit 100%  $bc_1$  complex activity, whereas isolated  $bc_1$  complex is only 50% active. The absence of the cleaved octapeptide might impair structural integrity in a manner that indirectly affects catalytic activity but does not cause loss of iron-sulfur protein from the complex.

In the purified  $bc_1$  complexes from the strains in which the MIP and MPP sites were blocked, we noticed reduced levels of subunit 10 (Fig. 3C). The amount of subunit 10 was also decreased, but to a lesser extent, in the  $bc_1$  complex from the S183C mutant. In the crystal structure of the bovine enzyme, the homologous subunit interacts with the iron-sulfur protein and is peripherally located (30, 31). Taken together these results suggest that subunit 10 is added after i-ISP is processed to m-ISP.

To determine whether the iron-sulfur cluster must be inserted before i-ISP is processed to m-ISP, we included a mutant in which the iron-sulfur cluster is not properly inserted but the apoprotein is stably assembled into the complex (21). The ratio of m-ISP to i-ISP in isolated  $bc_1$  complex or mitochondrial membranes from the S183C mutant as judged by Coomassie blue staining is diminished compared with the ratio in the membranes from wild type yeast. Therefore we conclude that although the iron-sulfur cluster normally is inserted into i-ISP, the processing from i-ISP to m-ISP does not require cluster insertion, but either the processing is slowed in the absence of cluster insertion or m-ISP is less stable without the cluster.

In previous experiments, Fu and Beattie (5) imported iron-sulfur protein into *S. cerevisiae* mitochondria *in vitro* and precipitated the radioactively labeled protein with antibodies specific to iron-sulfur protein or with antibodies that had been raised against  $bc_1$  complex but did not recognize iron-sulfur protein. After *in vitro* import of iron-sulfur protein and extraction of the mitochondria with detergent, both antibodies precipitated m-ISP. When MIP processing of i-ISP to m-ISP was inhibited by addition of EDTA and *o*-phenanthroline, the antibodies against iron-sulfur protein precipitated i-ISP, whereas the antibodies raised against  $bc_1$  complex did not. From this it was concluded that m-ISP but not i-ISP could be assembled into the  $bc_1$  complex.

We performed similar experiments, but instead of blocking the processing of the presequence with metal chelators we imported iron-sulfur proteins with modified presequences in which the MPP or MIP recognition sites had been destroyed. Our data show that i-ISP is assembled with other components of the  $bc_1$  complex in these mutant strains and that even in mitochondrial membrane extracts from wild type yeast a significant amount of i-ISP can be precipitated with antibodies against  $bc_1$  complex.

Boumans and co-workers (26, 27) showed that *o*-phenanthroline binds to solubilized  $bc_1$  complex and disrupts the iron-sulfur cluster. This can explain our finding that coimmunoprecipitation of iron-sulfur protein with the  $bc_1$  complex in the presence of *o*-phenanthroline is much less efficient, if after *o*-phenanthroline has disrupted the iron-sulfur cluster the protein is less stably associated with the complex. In addition, we have shown previously that high concentrations of EDTA and *o*-phenanthroline not only block processing of the iron-sulfur protein but also inhibit import of the precursor protein into the mitochondrial matrix (24). Either of these *o*-phenanthroline effects might explain why Fu and Beattie (5) were unable to detect i-ISP associated with the  $bc_1$  complex after they inhibited MIP with *o*-phenanthroline.

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