

## Two-step Processing Is Not Essential for the Import and Assembly of Functionally Active Iron-Sulfur Protein into the Cytochrome *bc*<sub>1</sub> Complex in *Saccharomyces cerevisiae*\*

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**The iron-sulfur protein of the cytochrome *bc*<sub>1</sub> complex is one of a small number of proteins that are processed in two sequential steps by matrix processing peptidase (MPP) and mitochondrial intermediate peptidase (MIP) during import into *Saccharomyces cerevisiae* mitochondria. To test whether two-step processing is necessary for import and assembly of the iron-sulfur protein into the cytochrome *bc*<sub>1</sub> complex, we mutagenized the presequence of the iron-sulfur protein to eliminate the original MPP site and replace the MIP site with a new MPP site. The mutated presequence is cleaved and forms mature-sized protein in a single step, and the mature-sized iron-sulfur protein is correctly targeted to the outer side of the inner mitochondrial membrane *in vitro*.**

**Mutant iron-sulfur protein which is processed to mature size in one step complements the respiratory deficient phenotype of a yeast strain in which the endogenous gene for the iron-sulfur protein is deleted. These results establish that mature-sized iron-sulfur protein can be formed by single-step processing and assembled into a functionally active form in the cytochrome *bc*<sub>1</sub> complex in *S. cerevisiae*.**

The majority of mitochondrial proteins are encoded in the nucleus, translated on cytosolic ribosomes, and then targeted to the mitochondria by amino-terminal leader presequences (1–3). During or after translocation of the precursor proteins into the mitochondria, the presequences are usually cleaved by specific proteases. Most presequences are removed in one step by matrix processing peptidase (MPP)<sup>1</sup> (4). However, some presequences are removed in two sequential steps in which initial cleavage by MPP results in an intermediate form, which is then processed by mitochondrial intermediate peptidase (MIP) to yield the mature protein (5). In this second step MIP removes exactly eight amino acids from the amino terminus of the intermediate. No consensus sequences for the two proteases have been found, but an arginine residue is often found at position –2 from the MPP cleavage site (6–8). The octapep-

tides that are removed by MIP always contain a hydrophobic residue at position –8 and a small residue such as glycine, serine, or threonine at position –5 relative to the amino terminus of the mature protein (6–8).

The biological function of the intermediate octapeptide and the reason for processing of some precursors in two steps are not fully understood. This question is especially intriguing, since identical proteins in different species are sometimes processed in different ways. The iron-sulfur protein (ISP) of the cytochrome *bc*<sub>1</sub> complex is processed in two steps by MPP and MIP during import into *Saccharomyces cerevisiae* mitochondria (9), whereas the iron-sulfur protein of bovine heart mitochondria is processed in only one step by MPP (10). One hypothesis to explain the need for two-step processing is that the amino terminus of a twice-cleaved precursor is structurally incompatible with cleavage by MPP and that the octapeptides function as spacers to satisfy structural requirements for processing (11).

To examine the role of the octapeptide-containing intermediate in targeting and function of the iron-sulfur protein of *S. cerevisiae*, we mutagenized the presequence to convert sequential two-step processing by MPP and MIP into one-step processing by MPP only. Here we report that mature-sized iron-sulfur protein is generated in one cleavage step by MPP, assumes its correct place on the outer side of the inner mitochondrial membrane, and is functional *in vivo*. This means that the amino terminus of the mature protein is structurally compatible with single-step processing by MPP, and that two-step processing is not necessary for correct targeting and assembly of iron-sulfur protein into the cytochrome *bc*<sub>1</sub> complex in the inner mitochondrial membrane of *S. cerevisiae*.

### 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 from ICN. EDTA was from Fisher and *o*-phenanthroline from Sigma. Automated sequencing was performed using the Dye Deoxy Terminator Cycle Sequencing Kit from Applied Biosystems Inc.

**Isolation of Mitochondria for *in Vitro* Import of Iron-Sulfur Protein**—Yeast strain W303-1A was grown in 1% yeast extract, 2% peptone, 2% galactose (YPGal) to an optical density at 600 nm of 2–4. Mitochondria were isolated from spheroplasts and frozen as described previously (12). Immediately before use mitochondria were thawed at room temperature and divided into 0.2-ml aliquots. To each aliquot 1 ml of ice-cold buffer containing 0.6 M mannitol, 20 mM Hepes-KOH, pH 7.4, 0.1% bovine serum albumin, 0.5 mM magnesium acetate was added, and mitochondria were reisolated by centrifugation for 5 min at 16,000 × *g* at 4 °C. They were then resuspended in an appropriate volume of the same buffer.

**Western Analysis of Mitochondrial Membranes**—For detecting iron-sulfur protein in mitochondrial membranes by Western blot analysis, yeast cells were broken with a glass bead beater, and mitochondrial membranes were isolated as described previously (13). Mitochondrial membranes were resolved by SDS-PAGE (14), and iron-sulfur protein

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

and cytochrome  $c_1$  were detected by Western blotting (15), using monoclonal antibodies to the iron-sulfur protein and cytochrome  $c_1$  (16).

**Import of Iron-Sulfur Protein into Mitochondria *in Vitro***—The *in vitro* import mixture contained 9% (v/v) translated precursor in rabbit reticulocyte lysate and an additional 13% (v/v) of untranslated lysate. It 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 30 μg of mitochondrial protein in a total volume of 0.1 ml. Prior to the addition of radioactive precursor, the import mixture was kept on ice for 5 min to energize the mitochondria. To obtain deenergized mitochondria, a sample was incubated for 5 min on ice in the presence of 22 μM antimycin, 20 μg/ml valinomycin, and 20 μg/ml FCCP before addition of precursor. To all other samples, precursor was added, and import was performed for 20 min at 30 °C, while the deenergized mitochondria sample was kept on ice. Import was stopped by placing the samples on ice and adding antimycin, valinomycin, and FCCP to the concentrations indicated above.

**Formation of Mitoplasts and Proteinase K Treatment**—To disrupt the outer mitochondrial membrane, mitochondria were pelleted, resuspended in 0.2 ml of 20 mM Hepes, pH 7.4, and kept on ice for 25 min with gentle vortexing at 5-min intervals. Mitoplasts were then reisolated and resuspended in 0.1 ml of import buffer.

For proteinase K treatment, samples were divided in half, and while one half was kept on ice, the other half was incubated with 60 μg/ml proteinase K for 30 min on ice. Digestion was stopped by addition of phenylmethylsulfonyl fluoride to 2 mM and incubation on ice for 5 min. Samples were then pelleted, resuspended in 20 μl of SDS sample buffer, and then analyzed by SDS-PAGE and fluorography of the dried gels.

**Enzyme Assays**—Ubiquinol-cytochrome  $c$  oxidoreductase activity was assayed at 23 °C using 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinol as a substrate (17). Reduction of cytochrome  $c$  was monitored at 550 versus 539 nm on an Aminco DW-2a spectrophotometer in a dual wavelength mode.

***In Vitro* Transcription and Translation**—*In vitro* transcription using phage SP6 RNA polymerase and *in vitro* translation using rabbit reticulocyte lysate were performed according to supplier recommendations. Before use in the import experiment, polysomes were removed by centrifugation at 160,000 × *g* for 40 min. The DNA template had been linearized with *Scal* before transcription.

**Construction of Plasmids**—Site-directed mutagenesis was performed using the Clontech Transformer Mutagenesis Kit. The sequence of steps by which the site-directed mutations were introduced is summarized in Fig. 1. pJN4 was created by replacing the codon for the -10 arginine in the plasmid pGem3-RIP, carrying the gene for the iron-sulfur protein of *S. cerevisiae*, with the codon for glycine. pJN4 was then further mutagenized to give pJN5 by inserting the codons for arginine, leucine, and isoleucine after the codon for the +1 lysine. pJN6 was created in the same way as pJN5, except that the nucleotide sequence coding for arginine, leucine, and lysine was inserted after the +1 lysine. pJN7 was obtained from pJN4 by adding the codons for valine and arginine after the codon for the -1 serine and changing the codons for the +1 lysine and +3 threonine into the codons for tyrosine and histidine, respectively. pJN32 was obtained from pJN4 by changing the codon for the -11 lysine into the codon for glycine. In the same way pJN33 was obtained from pJN5, pJN34 from pJN6, and pJN35 from pJN7 (Fig. 1).

We encountered difficulties in performing site-directed mutagenesis of the iron-sulfur protein gene in the yeast expression vector pFL39:RIP, possibly because of secondary structure of the vector (*cf.* Wong and Komaromy (18)). We thus constructed the expression plasmids carrying the mutant iron-sulfur protein genes as follows. First the *Hind*III-*Pst*I fragment of YEP352-drRIP1, containing the complete promoter region and the first 208 nucleotides of the open reading frame of the iron-sulfur protein gene, was cloned into pGem-3 digested with the same enzymes. Site-directed mutagenesis was then performed on the pGem constructs, generating the same mutations as described above. The *Hind*III-*Pst*I fragments were then excised and directionally cloned between the *Hind*III-*Pst*I sites of pFL9:RIP, a single copy yeast expression vector containing the iron-sulfur protein gene. In this way the plasmids pJN38 (analogous to pJN32), pJN39 (analogous to pJN34), and pJN40 (analogous to pJN35) were obtained. All mutations were verified by sequencing the relevant coding regions.

## RESULTS

**Two-step Processing of Iron-Sulfur Protein Can Be Converted to One-step Processing by Moving the MPP Site to the Amino Terminus of the Mature Protein**—In order to clarify the role of two-step processing of the iron-sulfur protein of the cytochrome

$bc_1$  complex during import into yeast mitochondria, we mutagenized the presequence in such a way that the MPP cleavage site would be moved to the amino terminus of the mature protein as it is in the iron-sulfur protein of bovine heart mitochondria. For this purpose we first destroyed the existing MPP site at position -8 of the presequence as shown in Fig. 1.

The cleavage site for MPP is usually indicated by an arginyl residue at position -2 relative to the cleavage site, which is -10 relative to the amino terminus of the mature protein (6-8). We have previously shown that substitution of the -10 arginine in the presequence of yeast iron-sulfur protein by other amino acids has different effects on processing, depending on the amino acid substituted for the arginine (19). While substitution of the -10 arginine with lysine or alanine still allowed the major portion of iron-sulfur protein to be processed after import, substitution with glycine almost completely blocked maturation of iron-sulfur protein *in vitro*. However, upon longer exposure of the gels during autoradiography, a very small amount of mature iron-sulfur protein (m-ISP) could be detected. To further improve the inhibition of processing in the current study, we additionally substituted the -11 lysine with a glycine (Fig. 1). This residue has been shown to also play an important role in the recognition of presequences by MPP (20). When the double mutant pJN32 was imported into yeast mitochondria *in vitro*, no mature iron-sulfur protein could be detected even after 10 times longer exposure of the gels (Fig. 2).

The mutant in which the -10 arginine and -11 lysine at the original MPP site were both changed to glycines was then further mutagenized to create pJN33, in which there is a new MPP recognition site at the amino terminus of m-ISP as shown in Fig. 1. In mutant pJN33, the three-amino acid stretch "RLI" was inserted after the +1 lysine. This produces the "SKRLIS" amino acid motif that is found at the original MPP site and elongates the presequence by another three amino acids. It also changes the amino terminus of the mature protein from K to I. This change was made because it has been proposed that the amino terminus of a twice-cleaved precursor is incompatible with cleavage by MPP (11).

In mutant pJN34 the amino acid stretch "RLK" was inserted at the same position, thus creating a "SKRLKS" motif. This again adds three amino acids to the presequence, but keeps the amino terminus of the mature protein unchanged. This mutant sequence tests whether cleavage by MPP is incompatible with the original sequence at the amino terminus of the mature protein.

In mutant pJN35 we created a site similar to the "AS-VRYSH" motif found in the bovine iron-sulfur protein, which we previously showed is processed in only one step (10). This was done by inserting a valine and an arginine after the -1 serine and changing the +1 lysine and +3 threonine to tyrosine and histidine, respectively. Since MPP now cuts in front of the +1 serine, which was previously the +2 serine, the presequence is 33 amino acids, which is three longer than the wild-type presequence, and the mature protein is one amino acid shorter than the wild-type protein.

As shown by the time course of import in Fig. 2, the three mutant proteins encoded by pJN33, 34, and 35 are imported and processed efficiently *in vitro* and apparently at higher rates than the wild-type control, based on the amount of protease protected m-ISP formed. As expected, no intermediate length protein is observable at any time. The substitution of the amino-terminal lysine by isoleucine in pJN33 seems to have no effect, since both pJN33 and pJN34 are imported and processed at the same rate. Even pJN35, where only the -4 serine and -2 arginine are conserved compared to the site initially found in yeast, is imported and processed reasonably well.

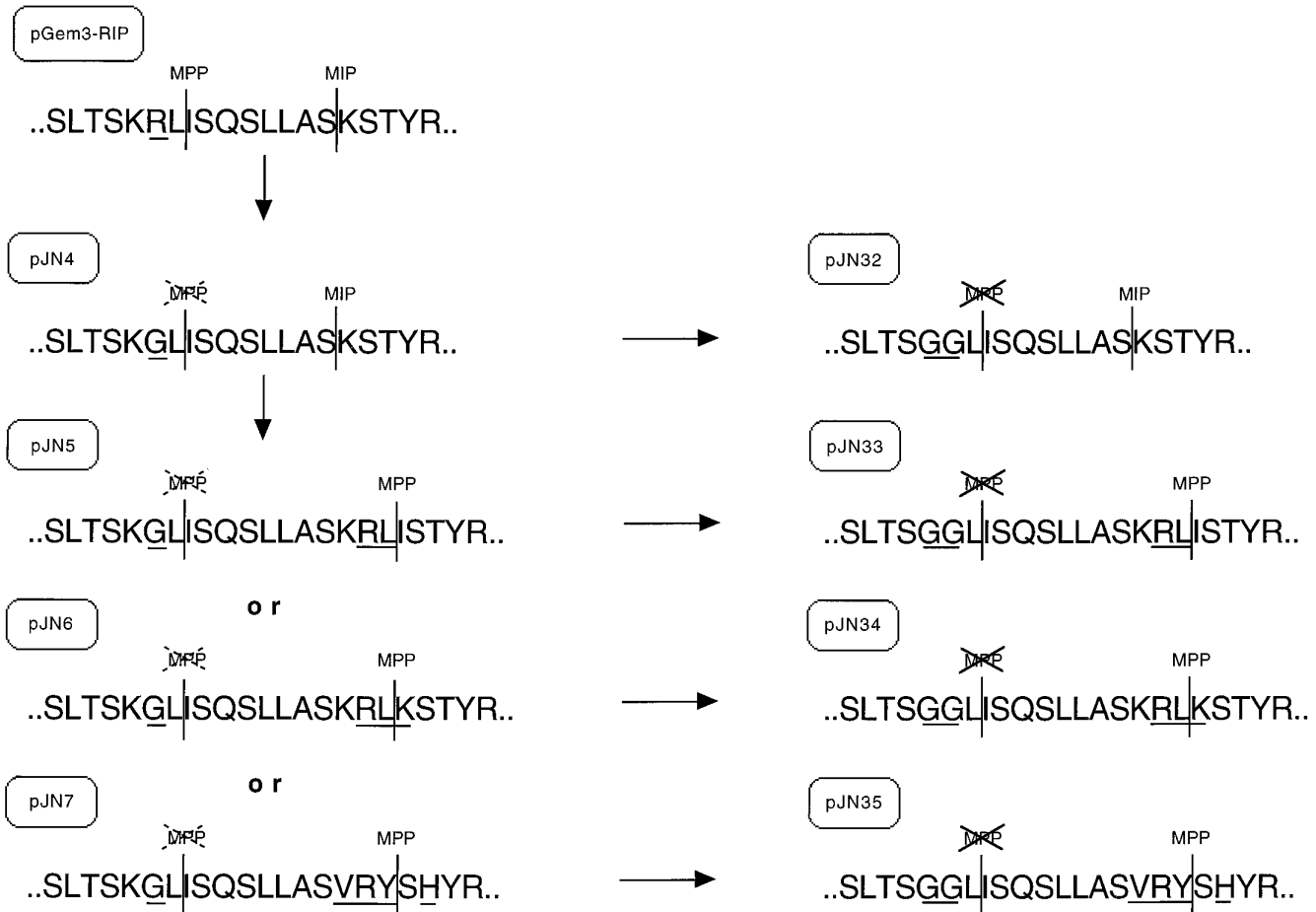


FIG. 1. **Construction of mutant iron-sulfur proteins.** The amino terminus of the mature, wild-type protein begins with the sequence KSTYR. The processing sites for MPP and MIP are indicated, and amino acids which were mutated or inserted are *underlined*.

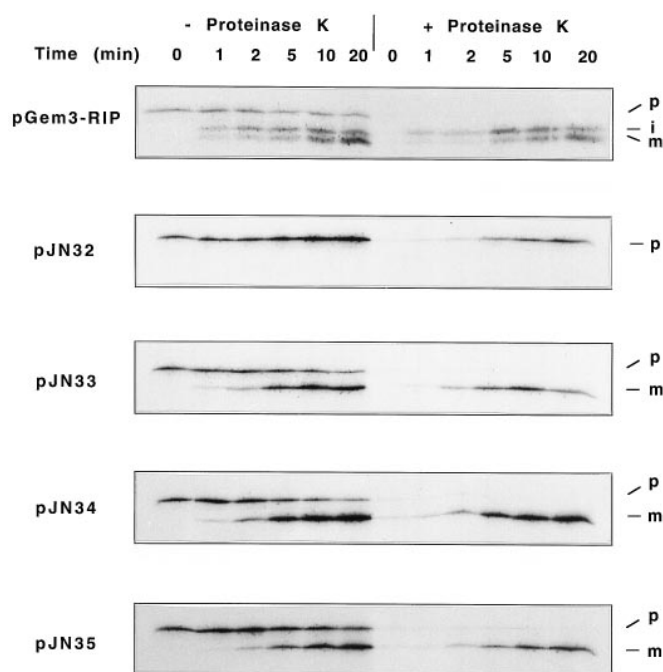
*One-step Processing Results in Cleavage of the Presequence at the Amino Terminus of the Mature Protein by MPP*—To distinguish whether MPP or MIP cleaves the presequence in mutants pJN33, 34, and 35, we took advantage of the fact that processing by MIP is inhibited completely by low concentrations of EDTA and *o*-phenanthroline, while import and processing by MPP are only slightly diminished (9, 19). When pJN34 was imported into yeast mitochondria that had been preincubated in buffer containing 10 mM EDTA and 2.5 mM *o*-phenanthroline, a treatment that completely abolishes MIP activity, protease protected m-ISP was formed as shown in Fig. 3.

The amount of m-ISP formed is less than in import experiments without metal chelators. This is due to the fact that the chelators also partially inhibit MPP and import itself, depending on their concentration (19). Formation of mature protein upon import under these conditions shows that the protease which removes the presequence of pJN34 in one step is not MIP. We thus conclude that, in mutants pJN33, 34, and 35, yeast MPP is able to cleave the iron-sulfur protein presequence in one step.

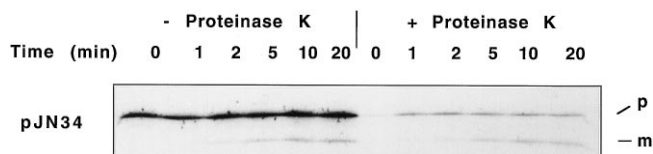
*Mutant Iron-Sulfur Protein That Is Processed in One Step Is Targeted to the Outer Side of the Inner Mitochondrial Membrane*—To determine whether the mutant iron-sulfur protein that is processed in one step is reexported from the matrix outward across the inner mitochondrial membrane, we examined the amount of m-ISP that was protected from proteinase K in mitoplasts. When wild-type iron-sulfur protein is imported, all of the mature protein is protected from proteinase K in intact mitochondria, as shown in Fig. 4. When mitochondria are converted to mitoplasts after the import, a significant

amount of mature protein becomes accessible to proteinase K, indicating that this portion has reached the outer side of the inner mitochondrial membrane. This is most obvious after 20 min of import. When pJN34 is imported under the same conditions, all of the mature protein is protease-protected in intact mitochondria, whereas in mitoplasts an amount comparable to the wild-type control is proteolyzed by proteinase K, indicating that this portion of the mutant protein is on the outer side of the inner mitochondrial membrane (Fig. 4).

*Mutant Iron-Sulfur Protein That Is Processed in One Step Is Functional in Vivo*—To test whether mutant iron-sulfur protein that is processed in one step is able to substitute for wild-type iron-sulfur protein *in vivo*, we performed the mutations corresponding to pJN34 and pJN35 (see Fig. 1) in a single copy yeast expression vector containing *RIP1*, which encodes the wild-type iron-sulfur protein. The resulting constructs, pJN39 and pJN40, were then used to transform JPJ1, a yeast strain in which the gene for the iron-sulfur protein has been deleted (21), thus creating yeast strains JN17 and JN18, respectively. JPJ1 is unable to grow on nonfermentable carbon sources due to the lack of iron-sulfur protein (21). As shown in Fig. 5, JN17 and JN18 were able to grow on ethanol/glycerol at the same rate as the deletion strain transformed with wild-type iron-sulfur protein, indicating that the mutant proteins were able to functionally substitute for wild-type iron-sulfur protein. When mitochondrial membranes were isolated from cultures grown in galactose, the ubiquinol-cytochrome *c* oxidoreductase activities of the membranes from JN17 and JN18 (0.65 unit/mg) were essentially identical to the activity of membranes from JPJ1 transformed with wild-type iron-sulfur protein gene



**FIG. 2. Time course of import into mitochondria of iron-sulfur proteins containing mutated presequences.** Wild-type iron-sulfur protein (*pGem3-RIP*) and mutant iron-sulfur proteins in which the processing site for MPP had been destroyed (*pJN32*) and new processing sites at the amino terminus had been constructed (*pJN33-35*) were imported into mitochondria for the indicated times. After import half of each sample was treated with proteinase K. The migration positions of precursor (*p*), intermediate (*i*), and mature (*m*) iron-sulfur protein are indicated. The presequences of the wild-type iron-sulfur protein, pGem3-RIP, and of the mutants, pJN32-pJN35, are shown in Fig. 1.



**FIG. 3. Time course of import into mitochondria of mutant iron-sulfur protein which is processed in one step in the presence of EDTA and *o*-phenanthroline.** Mutant iron-sulfur protein (*pJN34*) in which two-step processing had been converted into one-step processing was imported into mitochondria in the presence of 10 mM EDTA and 2.5 mM *o*-phenanthroline for the indicated times. After import half of each sample was treated with proteinase K. The migration positions of precursor (*p*) and mature (*m*) iron-sulfur protein are indicated.

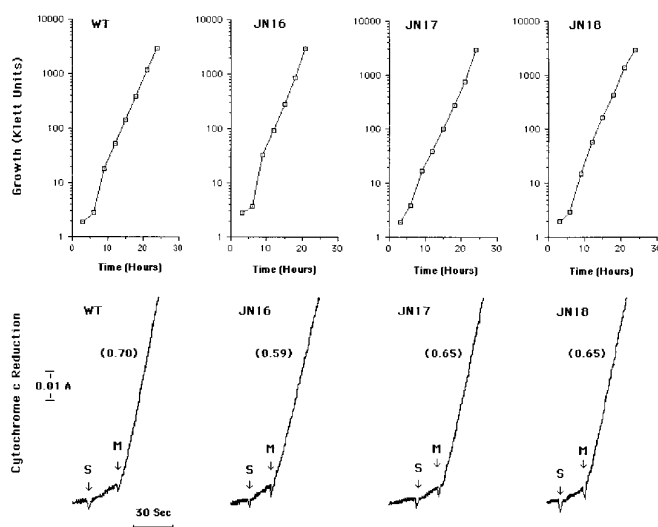
on the same vector (0.70 unit/mg, Fig. 5).

Whereas there is no immunodetectable iron-sulfur protein in mitochondrial membranes isolated from the deletion strain, as shown in Fig. 6, immunoblot analysis of membranes from JN17 and JN18 shows significantly increased amounts of m-ISP as compared to membranes from the wild-type strain. These results confirm the findings from the *in vitro* import experiments, that the mutant proteins are processed at a higher rate than wild-type protein. The immunoblots also reveal intermediate and mature forms of the wild-type protein, while only m-ISP is detected in the membranes from the mutants. The lack of an intermediate form in the mutants suggests that the one-step processing that was observed *in vitro* also occurs *in vivo*.

**Mutant Iron-Sulfur Protein in Which Processing Is Blocked *In Vitro* Is Processed Differently *In Vivo* and Also Rescues the Iron-Sulfur Protein-deficient Phenotype**—Simultaneous mutation of the -11 lysine and -10 arginine into glycines completely blocks processing of iron-sulfur protein by MPP *in vitro* (Fig. 2). To determine the effect of these mutations *in vivo*, we



**FIG. 4. Distribution of imported mutant iron-sulfur protein in mitochondria and mitoplasts.** Wild-type iron-sulfur protein (*pGem3-RIP*) and mutant iron-sulfur protein (*pJN34*) in which two-step processing had been converted into one-step processing were imported into mitochondria for the indicated times. After import portions of the mitochondria were converted into mitoplasts, and half of each sample was treated with proteinase K.



**FIG. 5. Effect of iron-sulfur proteins containing mutated presequences on growth rates and cytochrome *c* reductase activities of *S. cerevisiae*.** The four traces in the *top half* of the figure are growth rates of the indicated yeast strains on ethanol/glycerol at 30 °C. The four traces in the *bottom half* of the figure show reduction of cytochrome *c* versus time by mitochondrial membranes from the same strains. The nonenzymatic rate of reduction was measured by addition of 40  $\mu$ M decylubiquinol substrate (S), after which the enzymatic rate was initiated by addition of mitochondrial membranes (M). Numbers in parentheses alongside the cytochrome *c* reduction traces are rates of cytochrome *c* reduction, in micromoles  $\times$  min<sup>-1</sup>  $\times$  mg<sup>-1</sup>, which have been corrected for the nonenzymatic rate. WT is a yeast strain in which the *RIP1* gene is deleted and which was transformed with a single copy yeast expression vector carrying wild-type iron-sulfur protein. JN16 is a yeast strain in which the *RIP1* gene is deleted and which was transformed with a single copy yeast expression vector carrying mutant iron-sulfur protein in which the MPP processing site in the presequence was destroyed by replacing the -10 arginine and -11 lysine with glycines, which results in complete inhibition of processing *in vitro*. JN17 and JN18 are yeast strains in which the *RIP1* gene is deleted and which were transformed with a single copy yeast expression vector carrying mutant iron-sulfur protein gene with presequences equivalent to those in pJN34 and pJN35 (see Fig. 1), which are processed in one step *in vitro*.

also mutagenized the iron-sulfur protein on a single copy yeast expression vector in the same way. The resulting plasmid, pJN38, was used to transform JPJ1, thus creating JN16. Surprisingly, the mutant iron-sulfur protein in which processing is completely blocked *in vitro* also restored growth on ethanol/glycerol, and the growth rate of JN16 was essentially identical to that of JPJ1 transformed with the wild-type gene, as shown in Fig. 5. When mitochondrial membranes were isolated from

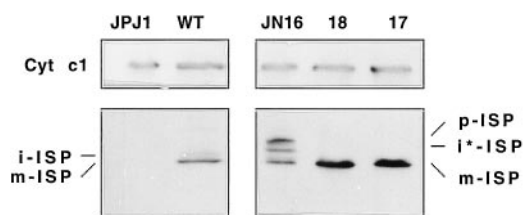


FIG. 6. Immunoblot analysis of mutant iron-sulfur proteins in mitochondrial membranes. Membranes from mitochondria containing wild-type and mutant iron-sulfur proteins were probed with antibodies against cytochrome  $c_1$  to demonstrate equal amounts of mitochondrial protein loaded onto the gel. The migration positions of precursor ( $p$ ), intermediate ( $i$ ), and mature ( $m$ ) iron-sulfur protein are indicated.  $i^*$  indicates a new intermediate form of the iron-sulfur protein that can only be detected in membranes derived from strain JN16. JPJ1 is the yeast strain in which the *RIP1* gene is deleted. WT, JN16, JN17, and JN18 are described in the legend to Fig. 5.

JN16 grown in galactose, the ubiquinol-cytochrome  $c$  oxidoreductase activity of the membranes was 80–85% of the activity of membranes from JPJ1 transformed with wild-type iron-sulfur protein gene (Fig. 5).

Immunoblot analysis of mitochondrial membranes from JN16 shows three major bands (Fig. 6). A significant amount of precursor iron-sulfur protein (p-ISP), which is usually not detectable in wild-type membranes, is detected in membranes from JN16. In addition, a novel intermediate form that runs at an apparently higher molecular weight than the normally observed i-ISP, and which we have designated  $i^*$ -ISP, is also detected in JN16 in addition to mature iron-sulfur protein. It is obvious that the mutations diminish the processing of iron-sulfur protein to such an extent that p-ISP can be detected in the mitochondrial membranes from this mutant. However, the amount of m-ISP formed is comparable to that formed in the wild-type membranes (Fig. 6), and this accounts for the ability of the strain to grow on the nonfermentable carbon sources. The formation of the novel intermediate form with an apparently higher molecular weight than the normal i-ISP also suggests that a processing step takes place at a site other than the usually observed MPP site. Whether this cleavage involves MPP or another protease and where in the presequence it occurs have not been determined.

#### DISCUSSION

A number of mitochondrial precursor proteins are processed sequentially by MPP and MIP following import into mitochondria while others are cleaved by MPP only. The iron-sulfur proteins of the cytochrome  $bc_1$  complexes of *S. cerevisiae* (22) and *Neurospora crassa* (23) belong to the first category, whereas the iron-sulfur protein of bovine heart mitochondria belongs to the second category (10). The reason why there is two-step processing of this protein in one species and one-step processing in another is not understood. The aim of our studies was to test whether two-step processing is necessary for import and assembly of functionally active iron-sulfur protein into the cytochrome  $bc_1$  complex in yeast.

One hypothesis regarding the function of the intermediate octapeptide was proposed by Isaya *et al.* (11), who constructed deletions in the octapeptide of the twice-cleaved precursor of human ornithine transcarbamylase and also exchanged leader sequences between once cleaved and twice-cleaved precursor proteins. Their studies led them to the conclusion that the mature amino terminus of a twice-cleaved precursor is structurally incompatible with MPP, and that the octapeptides have evolved to supply the structural requirements for cleavage.

To test this hypothesis and whether two-step processing is necessary, we constructed a mutagenized iron-sulfur protein,

which is processed to mature protein in only one step by MPP. We eliminated the existing MPP site in the presequence of the yeast iron-sulfur protein and then constructed a new MPP site at the amino terminus of the mature protein, which either resembled the previously existing MPP site in the yeast protein (pJN33 and pJN34) or the one found in the iron-sulfur protein of bovine heart mitochondria (pJN35).

pJN33 and pJN34 were employed to test whether the amino terminus of the iron-sulfur protein is incompatible with processing by MPP. While in pJN33 the amino terminus of the mature protein was changed from lysine to isoleucine to keep the SKRLIS motif, which is found in the original MPP site, in pJN34 the amino terminus of the mature protein was not changed. Both mutants were imported into mitochondria and processed to mature protein in a single step *in vitro*, and the two mutants appear to be processed to m-ISP at apparently the same rate and somewhat faster than the wild-type protein. These results indicate that the amino terminus of m-ISP is not incompatible with cleavage by MPP.

The third mutant that we tested (pJN35) contained an MPP site with the ASVRYSH motif found in the iron-sulfur protein of bovine heart mitochondria. This mutant was also processed in a single step, indicating that the amino terminus of the mature bovine protein is also compatible with cleavage by yeast MPP.

The difference between our results and those of Isaya *et al.* (11) might be due to the fact that they used chimeric fusion proteins in their experiments. It has been shown recently that cleavage of the presequence of fusion proteins is strongly influenced by the passenger protein (24), suggesting that the reason for lack of processing in the earlier studies (11) might not have been the incompatibility of the amino terminus with cleavage by MPP, but the incompatibility of the passenger protein with the presequence. However, it is possible that two-step processing is required for maturation of ornithine transcarbamylase, although this has not been tested with the native passenger protein. Likewise, our results do not bear on whether two-step processing is required for re-export from the matrix to the inner membrane, since the octapeptide at the amino terminus of i-ISP is thought not to be involved in targeting iron-sulfur protein from the matrix to the inner membrane (6).

In all of the mutants the existing MIP site was not destroyed by the mutations, therefore the possibility remained that MIP performed the single processing step. Three lines of evidence contradict this possibility, however. First, it has been shown previously that cleavage by MIP can only follow cleavage by MPP (25), and we found that there was no cleavage of the precursor protein in the pJN32 mutant, in which the MPP site was destroyed, but the MIP site was retained (Fig. 2). In addition, it is also known that MIP always removes an octapeptide from the amino terminus of a protein or peptide (26). And finally, we have shown that pJN34 is imported and processed in a single step in the presence of 10 mM EDTA and 2.5 mM *o*-phenanthroline, which completely block processing by MIP but which only partially block processing by MPP (19).

We also found that processing of mutant iron-sulfur protein in which the original MPP site is eliminated by replacement of the  $-10$  arginine and  $-11$  lysine with glycines is completely blocked *in vitro*, but this mutant iron-sulfur protein is processed to m-ISP by an alternative pathway *in vivo*. This alternative processing pathway generates a novel intermediate, which we designated as  $i^*$ -ISP, that migrates slower than i-ISP on SDS-PAGE gels. Although processing of p-ISP is retarded by eliminating the original MPP site, as evidenced by the accumulation of p-ISP in this mutant, the alternative processing which occurs in JN16 *in vivo* forms essentially the same

amount of m-ISP as is present in the wild-type control (Fig. 6), and thus the yeast grow on nonfermentable carbon sources. The alternative processing which forms m-ISP *in vivo* presumably occurs at such a low rate that it is not observable *in vitro*. However, the rate of processing by this alternative pathway is sufficient to keep pace with the rate of synthesis of other components of the cytochrome *bc*<sub>1</sub> complex, since the ubiquinol-cytochrome *c* reductase activity of the mitochondrial membranes from JN16 was the same as that of wild-type membranes.

The finding that mutant iron-sulfur protein which is processed to mature size in one step complements the respiratory deficient phenotype of a yeast strain in which the endogenous gene for the iron-sulfur protein is deleted shows that one-step processing generates iron-sulfur protein, which is functional *in vivo*. Although these results clearly demonstrate that two-step processing is not essential for import and formation of functional iron-sulfur protein in *S. cerevisiae*, they do not explain why two-step processing of this protein occurs in some species. One possible explanation is that two-step processing may regulate the rate of formation of m-ISP to match the rate of synthesis and assembly of other subunits of the cytochrome *bc*<sub>1</sub> complex. This explanation is consistent with the observation that when two-step processing is eliminated, the amount of m-ISP formed is far in excess of the amount of cytochrome *bc*<sub>1</sub> complex, as indicated by the amount of cytochrome *c*<sub>1</sub> in JN17 and JN18 (Fig. 6).

The formation of mature iron-sulfur protein in excess of that in the *bc*<sub>1</sub> complex as observed in JN17 and JN18 is not unprecedented. Nishikimi *et al.* (27) showed that there is approximately twice as much iron-sulfur protein in heart mitochondria as can be accounted for by cytochrome *bc*<sub>1</sub> complex, and Van Doren *et al.* (28) showed that when expressed from a high copy plasmid the iron-sulfur protein of the *bc*<sub>1</sub> complex can be assembled into the plasma membrane and the iron-sulfur cluster inserted in *Rhodobacter sphaeroides* in the absence of the other subunits of the complex. Whether the excess iron-sulfur protein in JN17 and JN18 contains iron-sulfur cluster remains to be determined.

The bovine iron-sulfur protein is processed only once, although in positions -17 to -8 it contains a RX(F/L/I)XX(T/S/G)XXXX motif that is typical for precursors that are cleaved sequentially by MPP and MIP (10). This suggests that one-step processing has evolved out of two-step processing and not the other way around. This evolution may have occurred to prevent futile trafficking of newly synthesized iron-sulfur protein to cytochrome *bc*<sub>1</sub> complexes, which already have a resident copy

of that protein. In bovine mitochondria, where one-step processing of the iron-sulfur protein has been demonstrated, the cleaved presequence stays as a subunit in the cytochrome *bc*<sub>1</sub> complex (10). If the iron-sulfur protein is the last subunit added to the cytochrome *bc*<sub>1</sub> complex (29), it could be competitively displaced by subsequently synthesized copies of iron-sulfur protein, unless there is a mechanism to prevent such displacement. The cleaved presequence is a different chemical entity than the attached presequence, but probably occupies the site in the complex that would otherwise be recognized by incoming p-ISP. In this manner newly synthesized iron-sulfur protein can discriminate between *bc*<sub>1</sub> complexes that have and complexes that do not have iron-sulfur protein.

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