THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 273, No. 15, Issue of April 10, pp. 8652–8658, 1998
Printed in U.S.A.

Processing of the Presequence of the *Schizosaccharomyces pombe* Rieske Iron-Sulfur Protein Occurs in a Single Step and Can Be Converted to Two-step Processing by Mutation of a Single Proline to Serine in the Presequence*

(Received for publication, August 29, 1997, and in revised form, January 14, 1998)

Jürgen H. Nett‡, Hermann Schägger§, and Bernard L. Trumpower‡¶

From the ‡Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and the §Universitätsklinikum Frankfurt, ZBC, Institut für Biochemie I, D-60590 Frankfurt, Germany

The iron-sulfur proteins of the cytochrome bc_1 complexes of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* contain the three amino acid motif R*X*(↓)(F/L/I)*XX*(T/S/G)*XXXX*(↓) that is typical for proteins that are cleaved sequentially in two steps by matrix processing peptidase (MPP) and mitochondrial intermediate peptidase (MIP). Despite the presence of this recognition sequence the *S. pombe* iron-sulfur protein is processed only once during import into mitochondria, whereas the *S. cerevisiae* protein is processed in two steps. Import of *S. pombe* iron-sulfur protein in which the putative MIP or MPP recognition sites are eliminated by site-directed mutagenesis and import of iron-sulfur protein into mitochondria from yeast mutants that lack MIP activity indicate that one step processing of the *S. pombe* iron-sulfur protein is independent of those sites and of MIP activity. Sequencing of the mature protein obtained after import *in vitro* and of the endogenous iron-sulfur protein isolated from mitochondrial membranes by preparative 2D-electrophoresis shows that MPP recognizes a second site in the presequence and processing occurs between residues 43 and 44.

If proline-20 of the *S. pombe* presequence is changed into a serine, a second cleavage step is induced. Conversely, if serine-24 of the *S. cerevisiae* presequence is changed to a proline, the first cleavage step that is normally catalyzed by MPP is blocked, causing precursor iron-sulfur protein to accumulate. Together these results indicate that a single amino acid change in the presequence is responsible for one-step processing in *S. pombe versus* two-step processing in *S. cerevisiae*.

The Rieske iron-sulfur protein of the mitochondrial cytochrome bc_1 complex (1), like the majority of mitochondrial proteins, is encoded in the nucleus, translated on cytosolic ribosomes, and then targeted to the mitochondria by an amino-terminal presequence (2–4). During or after translocation of the precursor ISP[1] into the mitochondria the presequence is cleaved by specific proteases.

The iron-sulfur protein of bovine heart mitochondria is processed in one-step by MPP (5), whereas the iron-sulfur proteins of *Neurospora crassa* (2) and *Saccharomyces cerevisiae* (6) are processed in two sequential steps, in which MPP removes the first part of the presequence from precursor ISP to form intermediate iron-sulfur protein, after which MIP removes an octapeptide to generate mature iron-sulfur protein (m-ISP). Why the presequence of the iron-sulfur protein is removed in two steps in some species and one step in others, and what properties of the presequence or the processing machinery determine two-step or one-step processing are not known.

Although no consensus sequences recognized by the two proteases have been found, precursors that are cleaved in two steps are characterized by a three amino acid motif R*X*(↓)(F/L/I)*XX*(T/S/G)*XXXX*(↓)[2] at the carboxyl terminus of their presequences (7, 8, 10). Recently the gene for the iron-sulfur protein of *Schizosaccharomyces pombe* has been cloned (9). Comparison of the presequences of the *S. pombe*, *S. cerevisiae* and *N. crassa* iron-sulfur proteins indicates that all three proteins have the three amino acid motif characteristic for two-step processing. However, upon *in vitro* import of the *S. pombe* iron-sulfur protein, it is processed to m-ISP in only one step. In the present study we have investigated the basis for the difference between one-step processing in *S. pombe* and two-step processing in *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^{35}S-label (methionine) from ICN. EDTA was from Fisher and *o*-phenanthroline and lysing enzymes from *Trichoderma harzianum* were from Sigma. Zymolyase was from ICN. Automated sequencing was performed using the Dye Terminator Sequencing Kit from Applied Biosystems Inc.

Isolation of Mitochondria—*S. cerevisiae* strains W303-1A and Y6040 (11) were grown in YPD (1% 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 (12).

S. pombe mitochondria were isolated essentially as described previously (13, 14). Haploid colonies of strain 972 (mating type h^-) were grown in 4 liters of Bacto™ YM broth (DIFCO) to late exponential phase. Cells were harvested at 2,000 × *g* for 5 min, washed twice in distilled water and incubated at 0.5 g/ml in 0.5 M β-mercaptoethanol,

* This work was supported by National Institutes of Health Grant GM 20379 and by a fellowship from the Deutsche Forschungsgemeinschaft (to J. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Dartmouth Medical School, 7200 Vail, Hanover, NH 03755. Tel.: 603-650-1621; Fax: 603-650-1389.

[1] The abbreviations used are: ISP, iron-sulfur protein; MPP, matrix-processing peptidase; MIP, mitochondrial intermediate peptidase; m-ISP, mature iron-sulfur protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[2] Arrows indicate the peptidase cleavage sites.

This paper is available on line at http://www.jbc.org

This is an Open Access article under the CC BY license.

0.1 M Tris-HCl, pH 9.3, for 10 min at 30 °C. After sedimenting the cells at 2,000 × *g* for 5 min they were washed three times in 0.5 M KCl, 10 mM Tris-HCl, pH 7.0. They were then resuspended in 1.35 M sorbitol, 1 mM EGTA, 10 mM citrate/phosphate, pH 5.8, 2 mg/ml zymolyase and incubated at 30 °C for 15 min. Lysing enzymes from *T. harzianum* (Novozym™234) were then added to 2 mg/ml and incubation at 30 °C was continued until formation of spheroplasts was detected (usually 10 min or less). All subsequent steps were carried out at 4 °C. Spheroplasts were harvested by centrifugation at 400 × *g* for 10 min and washed three times in 0.75 M sorbitol, 0.4 M mannitol, 10 mM MOPS, pH 6.8, 0.1% bovine serum albumin.

The washed spheroplasts were resuspended at 0.15 g/ml in 0.65 M mannitol, 2 mM EGTA, 10 mM MOPS, pH 6.8, 0.5% bovine serum albumin (breaking buffer) and broken by homogenizing gently in a loose fitting Dounce homogenizer. Intact cells and debris were removed by centrifugation at 1,000 × *g* for 10 min, and mitochondria were collected by centrifugation at 17,700 × *g* for 10 min. The mitochondrial pellet was resuspended in 40 ml of breaking buffer, residual debris was removed by centrifugation at 1,000 × *g* for 10 min, and mitochondria were obtained by centrifugation of the supernatant at 12,000 × *g* for 10 min and frozen as described previously (12).

Import of Iron-Sulfur Protein into Mitochondria in Vitro—The *in vitro* import mixture contained 4–9% (v/v) translated iron-sulfur protein precursor in rabbit reticulocyte lysate and an additional 13–18% (v/v) of rabbit reticulocyte lysate. The import mixture also contained 154 mM sucrose, 49 mM KCl, 7 mM MOPS-KOH, pH 7.2, 2.1% bovine serum albumin, 1.4 mM $MgCl_2$, 1 mM ATP, 4 mM NADH, and 20–40 μg of mitochondrial protein in a total volume of 0.1 ml. When *S. pombe* mitochondria were used or when import was performed in the presence of *o*-phenanthroline, the mixture was supplemented with an additional 1 M sorbitol. Prior to the addition of radioactive precursor the import mixture was kept on ice for 5 min to energize the mitochondria and to allow *o*-phenanthroline to penetrate the mitochondrial membranes. To obtain deenergized mitochondria, a sample was incubated for 5 min on ice in the presence of 20 μM antimycin, 20 μg/ml valinomycin, and 20 μg/ml carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone before addition of precursor. To all other samples, precursor was added and import was performed for 20 min or the indicated times at 30 °C while the deenergized mitochondria were kept on ice. Import was stopped by placing the samples on ice and adding antimycin, valinomycin, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to the concentrations indicated above. Proteinase K treatment was used to assess the extent of import (15).

Isolation of Mitochondrial Membranes—A 40-liter culture of *S. pombe* was grown to late exponential phase, the cells were sedimented by centrifugation, washed once with distilled water, and once with disruption buffer (0.1 M Tris-HCl, pH 8.0, 0.25 M sorbitol, 5 mM $MgCl_2$, 0.15 M potassium acetate, 1 mM dithiothreitol). The pellet was resuspended in a total volume of 30 ml of disruption buffer and frozen by slowly pouring as a thin stream into liquid nitrogen. The frozen cells were blended in liquid nitrogen for a total of 5 min at 1 min intervals in a stainless steel Waring blender. Additional liquid nitrogen was periodically added to prevent thawing of the cells. The lysed cell powder was thawed under warm water, and diisopropyl fluorophosphate was added to a final concentration of 1 mM. The cell debris was removed by centrifugation at 3000 × *g* for 10 min, and the resulting supernatant was centrifuged at 20,000 × *g* for 30 min to sediment the mitochondrial membranes. The membranes were washed twice in B1 (50 mM Tris acetate, pH 8.0, 0.4 M mannitol, 2 mM EDTA, 1 mM diisopropyl fluorophosphate), once in B2 (50 mM Tris acetate, pH 8.0, 0.15 M potassium acetate, 2 mM EDTA) and stored in B2 + 50% glycerol at −20 °C.

In Vitro Transcription and Translation—*In vitro* transcription using

Plasmid	Amino Acid Sequence
S. pombe	(13, 20, 30, 40; MPP ↓ after T)
pJN36	...SSLRRLLPVSSTASSLKGSMMTIPKFTSIRT YTDS..
pJN43	...SSLRRLL**S**VSSTASSLKGSMMTIPKFTSIRT YTDS..
pJN44	...SSL**GG**LLPVSSTASSLKGSMMTIPKFTSIRT YTDS..
pJN50	...SSLRRL**G**PV**F**STASSLKGSMMTIPKFTSIRT YTDS..
PJN92	...SSLRRLLPVSSTASSLKGSMMTIPKFTS**GG**T YTDS..
PJN93	...SSLRRLLPVSSTASSLKGS**AA**TIPKFTSIRT YTDS..
S. cerevisiae	(MPP ↓ 23; MIP ↓ 31)
pGEM3-RIP	...LTSKRL ISQSLLAS KSTY..
pJN4	...LTSK**G**L ISQSLLAS KSTY..
pJN32	...LTS**GG**L ISQSLLAS KSTY..
pJN42	...LTSKRL I**P**QSLLAS KSTY..
pJN49	...LTSKRL **G**SQ**F**LLAS KSTY..

FIG. 1. **Plasmids and amino acid sequences of the mutagenized presequences used in this study.** pJN36 and pGEM3-RIP are the plasmids carrying wild-type *S. pombe Rip1* and *S. cerevisiae RIP1* genes, respectively. Amino acids that contribute to the known and putative MPP and MIP recognition sites are *underlined* in the sequences of the wild-type genes, and the MPP and MIP processing sites are marked by *solid arrows* (↓). Amino acids that were introduced by site-directed mutagenesis are printed in *boldface letters*. The sequences are numbered from the NH_2-terminal methionines. The plasmids pGEM3-RIP (21), pJN4 (21), and pJN32 (15) were described previously. The remaining plasmids were constructed as part of this study.

Phage SP6 RNA polymerase and *in vitro* translation using rabbit reticulocyte lysate were performed according to supplier recommendations. In later experiments the TnT® coupled reticulocyte lysate system was used. Before use in the import experiment, polyribosomes were removed by centrifugation at 160,000 × *g* for 40 min.

Gel Electrophoresis—After *in vitro* import samples were analyzed on 15% SDS-PAGE gels, and protein bands were visualized by autoradiography (16). Blue native-PAGE and 2D-electrophoresis of proteins from mitochondrial membranes of *S. pombe* was performed as described previously (17). Cytochrome bc_1 complex was solubilized from mitochondrial membranes using a Triton X-100/protein ratio of 1.5. Linear 3.5–13% acrylamide gradient gels were used for Blue native-PAGE, and 13% acrylamide gels for Tricine/SDS-PAGE in the second dimension (18). The band of bc_1 complex, which was visible during Blue native-PAGE was excised from a preparative gel and cut into 4 pieces. A stack of these 4 pieces was processed by Tricine/SDS-PAGE in a second dimension and electroblotted onto Immobilon P membranes (19). A 473A protein sequencer (Applied Biosystems) was used for amino-terminal sequencing of electroblotted proteins.

Subcloning and Site-directed Mutagenesis of Iron-Sulfur Protein Genes—The *S. pombe* iron-sulfur protein gene was amplified by polymerase chain reaction using pFL61-SpISP (9) as template. The sense primer was 5′-TAATAAAAGCTTAGTAATTTAGACCGAATATTTTC-3′ (introducing a *Hin*dIII site at the 5′-end) and the antisense primer was 5′-TAATAAGAATTGTATTTATCCGATGATAATTTTG-3′. The amplified product was purified and subcloned into the TA cloning™ Vector, introducing an *Eco*RI site at the 3′-end. The *Hin*dIII-*Eco*RI fragment was isolated and cloned into a *Hin*dIII-*Eco*RI-digested pGEM-3 plasmid, creating pJN36. Site-directed mutagenesis was performed using the CLONTECH Transformer mutagenesis kit. The mutations that were introduced into the *S. pombe* and *S. cerevisiae* genes were verified by sequencing the relevant coding regions and are compiled in Fig. 1, along with the names of the corresponding plasmids.

Radiosequencing—After *in vitro* transcription and translation, the wild-type *S. pombe* iron-sulfur protein was imported into *S. pombe* mitochondria using 22% (v/v) translated precursor in 0.3 ml of *in vitro* import mixture. Following import the mitochondria were treated with proteinase K and phenylmethylsulfonyl fluoride as described (15), and proteins were separated by SDS-PAGE using 50 μl of sample for each of six lanes. They were then transferred to Pro Blott™ membranes using 10 mM CAPS, pH 11, 10% methanol as transfer buffer. After drying between blotting paper the bands corresponding to m-ISP were excised from six lanes and subjected to NH_2-terminal sequencing in an Applied Biosystems Protein Sequencer (model 476A).

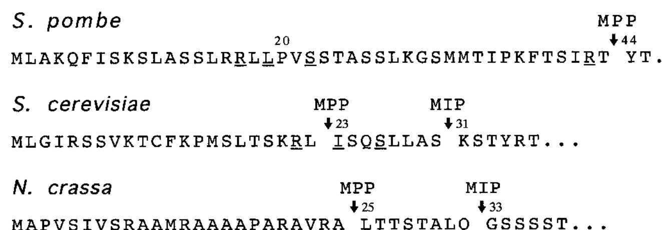


FIG. 2. **Comparison of amino acid presequences of the *S. pombe*, *S. cerevisiae*, and *N. crassa* Rieske iron-sulfur proteins.** The sequences are numbered from the amino terminus as in Fig. 1. The known MPP and MIP processing sites are indicated by *solid arrows* (↓), and amino acids that are thought to contribute to the MPP and MIP recognition sites are *underlined*.

RESULTS

S. pombe Iron-Sulfur Protein Is Processed in One Step When Imported into S. pombe or S. cerevisiae Mitochondria—Mitochondrial precursor proteins that are cleaved in two sequential steps by MPP and MIP share a highly conserved three amino acid motif R*X*(↓)(F/L/I)*XX*(T/S/G)*XXXX*(↓) at the carboxyl terminus of their leader sequences (7, 8, 10). The residue in position −11 from the final cleavage site also seems to be important for recognition of the precursor by MPP (20) and is often a basic amino acid. When we compared the deduced amino acid sequence of the iron-sulfur protein of *S. pombe* with the sequences for the homologous proteins of *S. cerevisiae* and *N. crassa* which are both processed in two steps by MPP and MIP, we found that the *S. pombe* protein also contained the three amino acid motif typical of sequential cleavage by MPP and MIP (Fig. 2). The *S. pombe* protein also contains a basic residue (Arg-16) at position −11 from the predicted final cleavage site between Ser-26 and Ser-27.

To determine whether the *S. pombe* iron-sulfur protein is indeed processed in two steps, we subcloned the open reading frame of the gene into a transcription vector and, after *in vitro* transcription and translation, imported the labeled protein *in vitro* into *S. pombe* mitochondria. As opposed to what was expected from the sequence comparison, no intermediate length protein is observable at any time during a time course of import (Fig. 3*A*). To check whether the predicted two-step cleavage motif is sufficient to direct sequential processing by

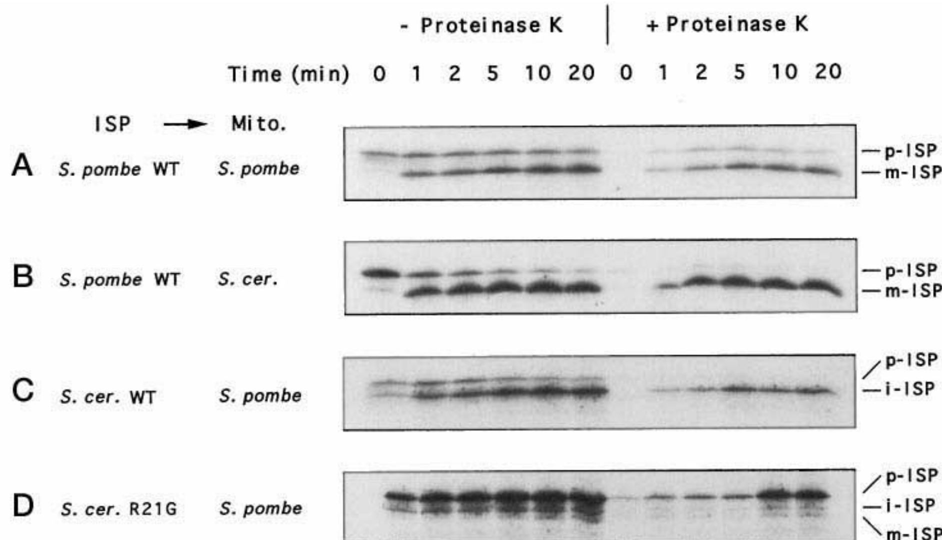


FIG. 3. **Import of *S. pombe* and *S. cerevisiae* iron-sulfur proteins into mitochondria.** S. pombe wild-type iron-sulfur protein was imported into *S. pombe* (*A*) and *S. cerevisiae* (*B*) mitochondria. *S. cerevisiae* wild-type iron-sulfur protein (*C*) and *S. cerevisiae* iron-sulfur protein in which Arg-21 was changed to Gly (*D*) were imported into *S. pombe* mitochondria. After import for the times indicated, half of each sample was treated with proteinase K. Indicated on the *left* of each panel is the species of iron-sulfur protein and of the mitochondria into which the protein was imported. The migration positions of precursor (*p*), intermediate (*i*), and mature (*m*) iron-sulfur proteins are indicated on the *right*. In this and all of the subsequent *in vitro* import experiments, controls were included to show that deenergization of the mitochondria with valinomycin blocked import. The nonimported precursor was fully sensitive to proteinase K digestion (results not shown).

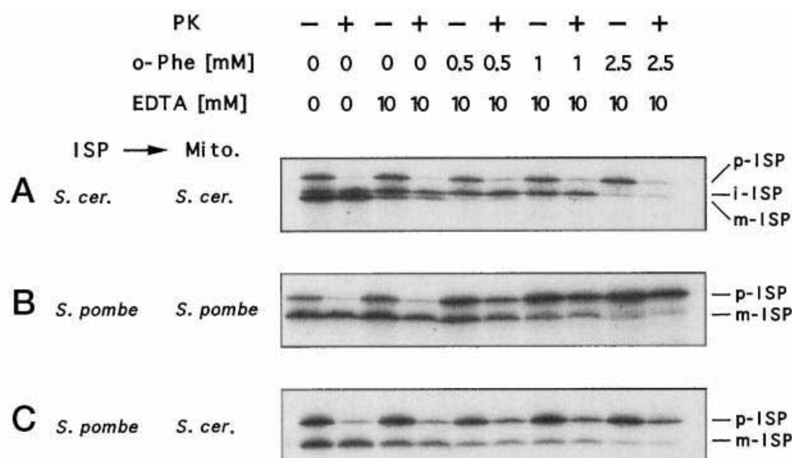


FIG. 4. **Import of iron-sulfur proteins in the presence of various amounts of metal chelators.** *S. cerevisiae* iron-sulfur protein was imported into *S. cerevisiae* mitochondria (*A*), and *S. pombe* iron-sulfur protein was imported into *S. pombe* (*B*) or *S. cerevisiae* (*C*) mitochondria for 20 min in the presence of the amounts of metal chelators indicated. After import, in this and subsequent experiments, half of each sample was treated with proteinase K (*PK*) to assess the extent of import.

MPP and MIP in *S. cerevisiae*, in which such processing of the endogenous iron-sulfur protein occurs (6, 15), we also imported the *S. pombe* iron-sulfur protein into *S. cerevisiae* mitochondria (Fig. 3*B*). Again only precursor and mature length protein is detectable, suggesting that the three amino acid motif is not sufficient by itself for sequential two-step processing.

To test whether *S. pombe* mitochondria contain any MPP and MIP activity, we imported *S. cerevisiae* iron-sulfur protein, which is normally processed by MPP and MIP in *S. cerevisiae* mitochondria, into *S. pombe* mitochondria (Fig. 3*C*). At first glance only intermediate length protein could be observed, but upon closer examination of the autoradiographs small amounts of mature length protein were detected. To show this more clearly, an altered *S. cerevisiae* iron-sulfur protein in which the processing by MPP was slowed down considerably by converting Arg-21 to Gly (21) was imported into *S. pombe* mitochondria. The altered precursor protein is processed very slowly to intermediate, therefore allowing precursor to accumulate inside the mitochondria. If the gel is then exposed for longer periods of time, as in Fig. 3*D*, it can be seen that mature length *S. cerevisiae* iron-sulfur protein can be formed in *S. pombe* mitochondria. The activity of the second protease seems to be much lower than in *S. cerevisiae,* since only trace amounts of mature protein are formed within a 20-min period. An alternative explanation could be, however, that the *S. pombe* protease has a specificity different than that of the *S. cerevisiae* protease and therefore recognizes and processes the *S. cerevisiae* iron-sulfur protein at a much lower rate. This also raises the possibility that the second cleavage in *S. pombe* might occur at a higher rate than the first, therefore making a possible intermediate hard to detect under standard import conditions.

Processing of the S. pombe Iron-Sulfur Protein Still Occurs in a Single Step When MIP Activity Is Inhibited by EDTA and o-Phenanthroline—MPP and MIP are metal-dependent proteases, and their activity can be blocked by addition of metal chelators EDTA and *o*-phenanthroline. In *S. cerevisiae* mitochondria MIP activity is blocked completely by 10 mM EDTA and 2.5 mM *o*-phenanthroline (6), whereas the concentrations sufficient to block MPP activity also abolish import of the precursor protein itself (21). To detect a possible intermediate, we inhibited the *S. pombe* processing peptidase(s) with increasing concentrations of metal chelators and imported *S. pombe* iron-sulfur protein into the mitochondria. Whereas it was confirmed that increasing concentrations of the chelators inhibit conversion of intermediate ISP to m-ISP when *S. cerevisiae* iron-sulfur protein was imported into *S. cerevisiae* mitochondria (Fig. 4*A*), no intermediate length protein was observable when the same concentrations of chelators were used to inhibit the peptidase activities during import of the *S. pombe* protein into the *S. pombe* mitochondria (Fig. 4*B*).

The processing peptidases of *S. pombe* mitochondria have thus far not been characterized, and the possibility remains that in contrast to *S. cerevisiae* inhibition of the second protease might require higher chelator concentrations, therefore making this approach not suitable for the detection of a possible intermediate. For this reason we also imported *S. pombe* iron-sulfur protein into *S. cerevisiae* mitochondria in which MIP had been inhibited (Fig. 4*C*). Again no intermediate was observable at any chelator concentration.

Mutations That Destroy the Putative MPP and MIP Sites Do Not Affect Processing of S. pombe Iron-Sulfur Protein—To clarify whether the putative MPP and MIP recognition sites are essential for processing of the *S. pombe* iron-sulfur protein, we destroyed each of these sites by site-directed mutagenesis. As a control we first mutagenized the *S. cerevisiae* iron-sulfur protein gene to destroy the MPP and MIP sites in that protein. We have shown previously that changing Lys-20 and Arg-21 to glycines, which are at positions −11 and −10 from the final cleavage site, results in complete inhibition of processing *in vitro* of *S. cerevisiae* iron-sulfur protein by MPP (15).

To destroy the MIP recognition site in the *S. cerevisiae* protein we changed Ile-23 and Ser-26, in positions −8 and −5 from the final cleavage site (see Fig. 2). A large hydrophobic amino acid is usually found in position −8. Substitution of this residue by glycine in the *S. cerevisiae* iron-sulfur protein resulted in partial inhibition of the second cleavage step and accumulation of intermediate ISP upon import into *S. cerevisiae* mitochondria. Inhibition of processing was not complete, however, and a small amount of m-ISP was formed (results not shown). To further improve the inhibition of processing we additionally changed Ser-26 at position −5 from the final cleavage site, where there is usually a small hydroxylated amino acid or a glycine, into a phenylalanine, a large nonhydroxylated amino acid. The combination of the two mutations, I23G and S26F (pJN49 in Fig. 1), abolished processing by MIP completely, so that iron-sulfur protein was only processed to intermediate length upon import into *S. cerevisiae* mitochondria as shown in Fig. 5*C*.

When we performed the mutations destroying the putative MPP or MIP site in the *S. pombe* iron-sulfur protein and imported the mutant proteins into either *S. pombe* or *S. cerevisiae* mitochondria, neither import nor processing was affected significantly (Fig. 5, *A* and *B*). This further suggests that the *S. pombe* iron-sulfur protein is not processed via an intermediate length form, and that the putative MPP and MIP recognition sites are not necessary for processing of the *S. pombe* protein.

We also imported the altered *S. cerevisiae* proteins into *S. pombe* mitochondria to see whether *S. pombe* contains process-

ing enzymes that recognize the known sites for MPP or MIP. As shown in Fig. 5*D* for the altered *S. cerevisiae* protein in which the processing site for MPP had been destroyed, processing was inhibited completely, suggesting that Arg-21 and Lys-20 are essential for cleavage of the *S. cerevisiae* protein by *S. pombe* MPP. Surprisingly, the combination of I23G and S26F mutations that abolished processing of the *S. cerevisiae* protein by MIP upon import into *S. cerevisiae* mitochondria improved processing of the *S. cerevisiae* protein to mature form when it was imported into *S. pombe* mitochondria (Fig. 5*D*). This suggests that either the *S. pombe* MIP has a specificity that differs from that of *S. cerevisiae* MIP or that the second cleavage step is performed by a different protease in *S. pombe* mitochondria.

Mutation of a Proline to Serine in the S. Pombe Iron-Sulfur Protein Presequence Results in Two-step Processing, Whereas Mutation of a Serine to Proline in the S. Cerevisiae Iron-Sulfur Protein Presequence Blocks the First Step of Two-Step Processing—In searching for an explanation as to why the potential MPP site in the *S. pombe* iron-sulfur protein was not recognized in either *S. pombe* or *S. cerevisiae* mitochondria, we noted the presence of a proline in position 20 of the presequence, which is at position +2 relative to the predicted MPP site (Fig. 2). In the *S. cerevisiae* iron-sulfur protein, a serine residue (Ser-24) is found at the equivalent position. Since replacement of a serine with a proline would have a marked effect on local secondary structure of the presequence proximal to the MPP cleavage site, we suspected that this sequence difference might account for the difference in processing of the two presequences.

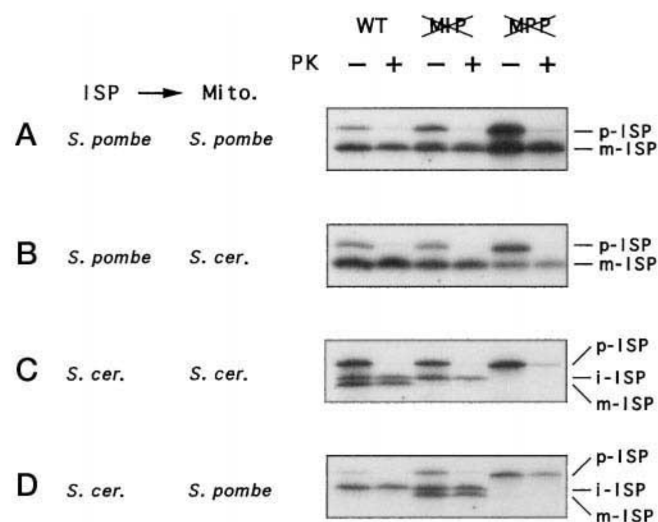


FIG. 5. **Import into mitochondria of iron-sulfur proteins containing altered presequences.** *S. pombe* and *S. cerevisiae* wild-type and altered proteins in which the processing sites for MIP (pJN49 and pJN50, see Fig. 1) or MPP (pJN32 and pJN44, see Fig. 1) had been destroyed were imported into *S. pombe* or *S. cerevisiae* mitochondria.

When we changed Pro-20 in the *S. pombe* iron-sulfur protein presequence to serine (creating pJN43), an additional intermediate length protein could be detected upon *in vitro* import into either *S. pombe* or *S. cerevisiae* mitochondria as shown in Fig. 6, *A* and *B*, respectively. The two processing steps that result seem to be independent, since m-ISP can also be formed in a single step. Although this does not demonstrate that this intermediate length protein is converted to m-ISP, there are numerous precedents that MPP will cleave very short presequences, and on this basis we infer that this is a *bona fide* intermediate in two-step processing. This does not preclude formation of m-ISP by an independent pathway in a single step as shown in Fig. 7.

Conversely, when Ser-24 in the *S. cerevisiae* iron-sulfur protein presequence was changed into a proline, the first step of the normal two-step processing of precursor ISP was blocked and only trace amounts of m-ISP or intermediate ISP and m-ISP could be detected upon import into *S. cerevisiae* or *S. pombe* mitochondria, respectively (Fig. 6, *C* and *D*). This result demonstrates that MPP cannot cleave efficiently when a proline is found at position +2 from the cleavage site.

Tyrosine-44 Is the Amino Terminus of the Mature S. pombe Iron-Sulfur Protein—After having established that the predicted protease recognition sites are not necessary for processing of the *S. pombe* iron-sulfur protein we determined the actual processing site by radiosequencing of the mature protein after import and processing of [^{35}S]methionine-labeled protein *in vitro*. As shown in Fig. 8*A* the methionine at position 50 is released in sequencing cycle seven, thus establishing Tyr-44 as the amino-terminal residue of the mature *S. pombe* iron-sulfur protein. To rule out that the observed radioactivity was due to

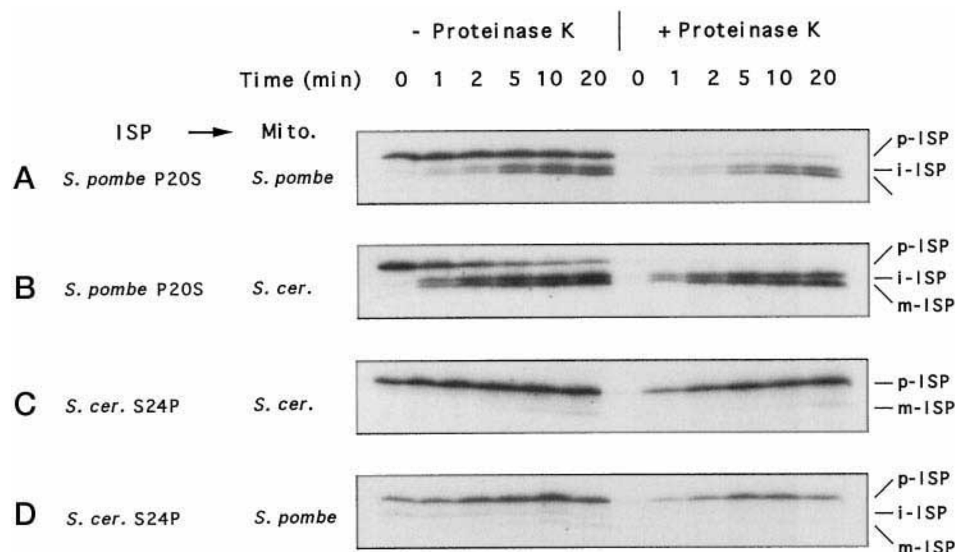


FIG. 6. **Import of iron-sulfur proteins with amino acid changes in position +2 from the proposed MPP cleavage site.** *S. pombe* iron-sulfur protein in which Pro-20 had been changed to serine (pJN43, see Fig. 1) was imported into *S. pombe* (*A*) or *S. cerevisiae* (*B*) mitochondria. *S. cerevisiae* iron-sulfur protein in which Ser-24 had been changed to proline (pJN42, see Fig. 1) was imported into *S. cerevisiae* (*C*) or *S. pombe* (*D*) mitochondria.

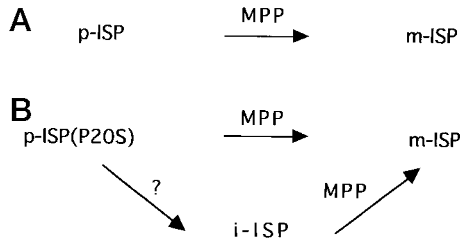


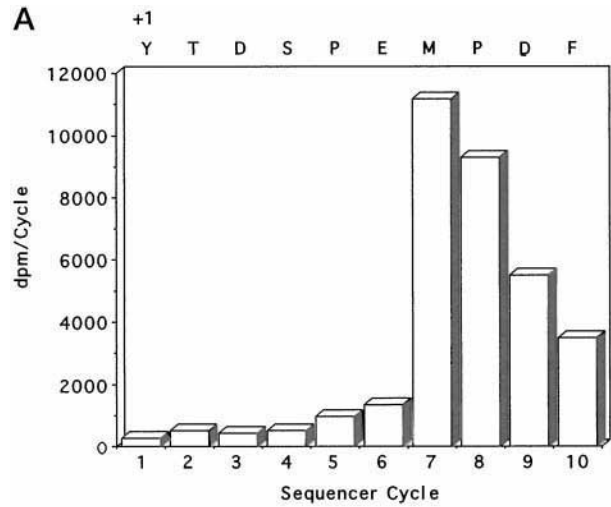
FIG. 7. **Model for the processing of the Rieske iron-sulfur protein in *S. pombe* and the changes in processing that result from changing Pro-20 in the presequence to serine.** The scheme in *panel A* shows the single-step processing of precursor ISP by MPP that forms m-ISP with Tyr-44 as the mature amino terminus. The scheme in *panel B* shows the two-step processing that results when Pro-20 is converted to Ser, and the possibility of forming m-ISP independently by two-step or one-step processing of the P20S form of the iron-sulfur protein.

release of methionines 32 and 33 in cycles seven and eight, we also performed radiosequencing on the mature form of an *S. pombe* iron-sulfur protein in which Met-32 and -33 had been changed to alanines by site-directed mutagenesis of the encoding DNA (pJN93). The same pattern for release of radioactivity as for the wild-type protein was observed, confirming the earlier findings (results not shown).

To verify our results *in vivo* we isolated mature iron-sulfur protein from *S. pombe* mitochondrial membranes by two-dimensional gel electrophoresis and subjected it to amino-terminal microsequencing. The first 10 amino-terminal residues were determined to be YTDSPEMPDF. This is consistent with the amino terminus of the mature iron-sulfur protein that had been generated *in vitro* (see Fig. 2).

Processing of S. pombe Iron-Sulfur Protein Is Probably Performed by MPP and Is Independent of MIP Activity—To determine which protease is responsible for the single-step cleavage of the *S. pombe* protein we changed the amino acids in positions −2 and −3 from the processing site to glycines by site-directed mutagenesis of the encoding DNA (creating pJN92; see Fig. 1). The arginine residue that is found in position −2 is typical for proteins that are processed by MPP, and we and others have shown that glycines at position −2 and −3 inhibit processing by MPP almost completely (21–23). When we imported the altered *S. pombe* protein encoded by pJN92 into *S. pombe* mitochondria *in vitro*, inhibition of processing could be observed when compared with the wild-type protein (Fig. 8*B*). We therefore conclude that MPP is most likely the protease that cleaves the *S. pombe* iron-sulfur protein in a single step.

To confirm that processing of the *S. pombe* iron-sulfur protein is independent of MIP activity we imported the wild-type *S. pombe* protein into mitochondria isolated from a yeast strain (Y6040) in which the gene for MIP has been disrupted (11). The *S. cerevisiae* iron-sulfur protein that was imported as a control is only processed to intermediate length form as expected, whereas the *S. pombe* wild-type protein is processed to mature length (Fig. 9). These results show clearly that single-step processing of the wild-type *S. pombe* protein is independent of MIP activity.



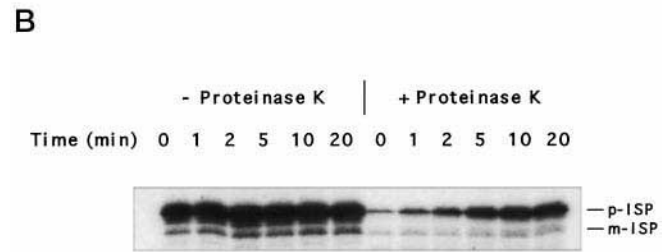


FIG. 8. **Determination of the amino terminus of the mature iron-sulfur protein of *S. pombe* and import into mitochondria of iron-sulfur protein with amino acid changes at positions −2 and −3 from the amino terminus of the mature protein.** In *panel A*, mature iron-sulfur protein of *S. pombe* was subjected to radiosequencing after *in vitro* import of [^{35}S]methionine-labeled protein as described under "Experimental Procedures." *+1* marks the amino terminus of the mature protein. The radioactivity in samples 8–10 is due to trailing from sample 7 and declines exponentially as expected. In *panel B*, *S. pombe* iron-sulfur protein in which Ile-41 and Arg-42, at the −2- and −3-positions relative to the mature amino terminus, had been changed to glycines (pJN92, see Fig. 1) was imported into *S. pombe* mitochondria.

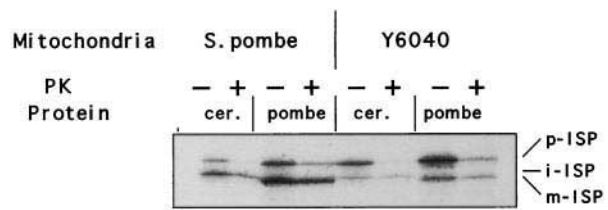


FIG. 9. **Import of wild-type iron-sulfur protein into mitochondria that lack MIP activity.** Wild-type *S. cerevisiae* iron-sulfur protein (*S. cer.*) and wild-type *S. pombe* iron-sulfur protein (*S. pombe*) were imported into mitochondria from yeast strain Y6040, which are devoid of MIP activity (11).

DISCUSSION

The iron-sulfur proteins of the bc_1 complexes of *S. cerevisiae* (6) and *N. crassa* (2) are processed sequentially by MPP and MIP upon import into mitochondria, whereas the iron-sulfur protein of beef heart mitochondria is cleaved once by MPP only (5). All three proteins contain the three amino acid motif R*X*(↓)(F/L/I)*XX*(T/S/G)*XXXX*(↓) that is thought to direct two-step processing, but the bovine protein also has an additional MPP site downstream, which is used as the cleavage site. The properties of this iron-sulfur protein that direct two-step processing in one species and one-step processing in another, and the reason why this protein would be processed in two steps in some species and in one step in others are not understood. The deduced amino acid sequence for the iron-sulfur protein of *S. pombe* (9) shows the three amino acid motif typical of processing by MPP and MIP, and therefore it had been proposed that this protein was cleaved twice (5).

The aim of our studies was to investigate the processing characteristics of the *S. pombe* iron-sulfur protein and to compare that processing to the processing of the homologous proteins in *S. cerevisiae* and bovine heart mitochondria. When the *S. cerevisiae* iron-sulfur protein is imported *in vitro,* intermediate and mature forms of the protein can easily be distin-

guished. Although the *S. pombe* iron-sulfur protein has a presequence very similar to the *S. cerevisiae* protein, our results clearly show that the *S. pombe* iron-sulfur protein is only processed in one step when imported into either *S. pombe* or *S. cerevisiae* mitochondria. To investigate whether *S. pombe* mitochondria contain MIP activity, we also imported *S. cerevisiae* iron-sulfur protein as a control. The protein was processed in two steps, albeit at a very low rate, suggesting that the *S. pombe* MIP either has a very low activity or an altered specificity. Another possible explanation is that an alternative protease cleaves at the MIP site unspecifically.

When we inhibited the processing proteases of *S. pombe* or *S. cerevisiae* mitochondria by increasing concentrations of metal chelators, no intermediate could be detected in either case, again suggesting only one step processing. In another approach to accumulate any possible intermediate, we destroyed the putative recognition sites for MPP and MIP by site-directed mutagenesis. We had shown before that changing Lys-20 and Arg-21 into glycines in the *S. cerevisiae* iron-sulfur protein (see Fig. 2) abolished *in vitro* processing by MPP completely, leading to accumulation of precursor (15). Here we have shown that the MIP site of the *S. cerevisiae* iron-sulfur protein can be destroyed by changing the large hydrophobic residue, Ile-23, at position −8 from the final cleavage site into glycine, and changing the small residue, Ser-26, at position −5 into phenylalanine. Those amino acid changes do not seem to block processing of the *S. cerevisiae* protein by MPP, since intermediate length iron-sulfur protein accumulates when imported into *S. cerevisiae* mitochondria *in vitro*. When we made the same changes in the *S. pombe* iron-sulfur protein and imported the *S. pombe* proteins into *S. pombe* or *S. cerevisiae* mitochondria, processing was not affected at all. This confirmed that processing of the *S. pombe* iron-sulfur protein occurs in one step and is independent of those sites.

In an attempt to determine the factors that prevent MPP and MIP from processing at the predicted sites, we changed Pro-20 in the *S. pombe* iron-sulfur protein, which is residue −7 from the predicted MIP cleavage site, into a serine and also changed Ser-24 in the *S. cerevisiae* iron-sulfur protein into a proline. Whereas the first mutation resulted in the formation of an additional processing step, the second one inhibited the processing of the altered protein almost completely. In a study that tried to identify new natural substrates for MIP, Isaya and co-workers (10) found that serine seems to be the preferred amino acid at position −7 from the MIP cleavage site. It is thought that the presequences of mitochondrial precursor proteins act by forming amphipatic helices (24), and it has been shown that helical elements in the presequence can be recognized by MPP (25). Proline on the other hand is a helix-breaking residue. When in position −7 of the proposed MPP/MIP recognition site proline seems to be a key residue, preventing the first and possibly also the second processing step from occurring. We also substituted the prolines in the MPP/MIP consensus motif (. . . RPLVASVSLNVPA . . .) in the bovine iron-sulfur protein (see Ref. 5) with leucine and serine, respectively, without any significant effect on processing upon import into *S. pombe* or *S. cerevisiae* mitochondria (results not shown). Ogishima *et al.* (26) have shown recently that a proline in the presequence of malate dehydrogenase is important for processing by MPP. This suggests that the function of the proline is dependent on its position within the consensus motif.

Because our results indicated that the *S. pombe* iron-sulfur protein was not processed at the predicted cleavage sites, we determined the amino terminus of the mature protein by radiosequencing of the *in vitro* imported protein and thus established that processing occurred before Tyr-44. We confirmed this result by also microsequencing mature *S. pombe* iron-sulfur protein that had been generated *in vivo*.

To determine whether MPP cleaves at the Tyr-44 site, we mutagenized the arginine and isoleucine residues that are found in positions −2 and −3 into glycines. It has been suggested that MPP from rat liver, *N. crassa*, or *S. cerevisiae* mitochondria can only cleave efficiently when an arginine is found in position −2. It has also been shown that glycine at this position almost completely prevents processing (21–23). The processing of the altered *S. pombe* protein was strongly inhibited, as would have been expected for MPP dependent cleavage. It therefore seems that processing of the *S. pombe* iron-sulfur protein is more closely related to that of the protein from bovine heart mitochondria than to processing of the iron-sulfur protein from *S. cerevisiae*. To our knowledge there has been no conclusive evidence for the functional relevance of two-step processing to date, and it is possible that during evolution the second processing step has become obsolete and has been substituted by one-step processing.

Acknowledgments—We thank Dr. Grazia Isaya for providing the MIP deletion strain Y6040.

REFERENCES

1. Yang, X., and Trumpower, B. L. (1986) *J. Biol. Chem.* **261,** 12282–12289
2. Hartl, F. U., Schmidt, B., Wachter, E., Weiss, H., and Neupert, W. (1986) *Cell* **47,** 939–951
3. Attardi, G., and Schatz, G. (1988) *Annu. Rev. Cell Biol.* **4,** 289–333
4. Pfanner, N., and Neupert, W. (1990) *Annu. Rev. Biochem.* **59,** 331–353
5. Brandt, U., Yu, L., Yu, C.-A., and Trumpower, B. L. (1993) *J. Biol. Chem.* **268,** 8387–8390
6. Fu, W., Japa, S., and Beattie, D. S. (1990) *J. Biol. Chem.* **265,** 16541–16547
7. Hendrick, J. P., Hodges, P. E., Rosenberg, L. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4056–4060
8. Gavel, Y., and von Heijne, G. (1990) *Protein Eng.* **4,** 33–37
9. di Rago, J.-P., Bruel, C., Graham, L. A., Slonimski, P., and Trumpower, B. L. (1996) *J. Biol. Chem.* **271,** 15341–15345
10. Branda, S. S., and Isaya, G. (1995) *J. Biol. Chem.* **270,** 27366–27373
11. Isaya, G., Miklos, D., and Rollins, R. A. (1994) *Mol. Cell. Biol.* **14,** 5603–5616
12. Guthrie, C., and Fink, G. R. (1991) *Methods Enzymol.* **194,** 631–638
13. Moore, A. L., Walters, A. J., Thorpe, J., Fricaud, A.-C., and Watts, F. Z. (1992) *Yeast* **8,** 923–933
14. Albury, M. S., Dudley, P., Watts, F. Z., and Moore, A. L. (1996) *J. Biol. Chem.* **271,** 17062–17066
15. Nett, J. H., Denke, E., and Trumpower, B. L. (1997) *J. Biol. Chem.* **272,** 2212–2217
16. Laemmli, U. K. (1970) *Nature* **227,** 680–685
17. Schägger, H., Cramer, W. A., and von Jagow, G. (1994) *Anal. Biochem.* **217,** 220–230
18. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379
19. Schägger, H. (1994) in *A Practical Guide to Membrane Protein Purification* (von Jagow, G., and Schägger, H., eds) pp. 76–79, Academic Press, Orlando, FL
20. Niidome, T., Kitada, S., Shimokata, K., Ogishima, T., and Ito, A. (1994) *J. Biol. Chem.* **269,** 24719–24722
21. Nett, J. H., and Trumpower, B. L. (1996) *J. Biol. Chem.* **271,** 26713–26716
22. Horwich, A. L., Kalousek, F., Fenton, W. A., Pollock, R. A., Rosenberg, L. E. (1986) *Cell* **44,** 451–459
23. Arretz, M., Schneider, H., Guiard, B., Brunner, M., and Neupert, W. (1994) *J. Biol. Chem.* **269,** 4959–4967
24. Von Heijne, G. (1986) *EMBO J.* **5,** 1335–1342
25. Sjöling, S., Eriksson, A.-C., and Glaser, E. (1994) *J. Biol. Chem.* **269,** 32059–32062
26. Ogishima, T., Niidome, T., Shimokata, K., Kitada, S., and Ito, A. (1995) *J. Biol. Chem.* **270,** 30322–30326