Dissociation of Import of the Rieske Iron-Sulfur Protein into _Saccharomyces cerevisiae_ Mitochondria from Proteolytic Processing of the Presequence*

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The correlation between the import of the Rieske iron-sulfur protein into the mitochondrial matrix and processing of the precursor protein by matrix processing peptidase was investigated using high concentrations of metal chelators and iron-sulfur protein in which the recognition site for the matrix processing peptidase was destroyed by site-directed mutagenesis. High concentrations of EDTA and o-phenanthroline inhibit import of iron-sulfur protein into the matrix. The non-chelating structural isomers m-phenanthroline and p-phenanthroline inhibit import similar to o-phenanthroline, indicating that inhibition of import is mainly independent of the metal chelating ability of the compounds.

Iron-sulfur protein in which the recognition site for the matrix processing peptidase had been destroyed by a point mutation was efficiently imported into the matrix space. Import of this mutant iron-sulfur protein was inhibited by the same concentrations of EDTA and o-phenanthroline, which inhibit import of the wild-type protein. These results indicate that import of the iron-sulfur protein into the mitochondrial matrix is independent of proteolytic processing of the presequence, and that o-phenanthroline together with EDTA inhibits import of iron-sulfur protein into the matrix space of mitochondria by inhibiting a step other than proteolysis of the presequence.

The Rieske iron-sulfur protein is an essential subunit of mitochondrial cytochrome _bc_ complexes and, like the majority of mitochondrial proteins, is encoded by a nuclear gene and synthesized on cytoplasmic ribosomes. The iron-sulfur protein is then post-translationally imported into the mitochondria where it is inserted into the _bc_ complex in the inner mitochondrial membrane (1).

During import and assembly of the iron-sulfur protein into _Neurospora crassa_ (1) and _Saccharomyces cerevisiae_ (2–4) mitochondria, a 30-amino acid amino-terminal targeting presequence is removed in two steps. A matrix processing peptidase (MPP) first removes a 22-amino acid peptide from the presequence of the precursor iron-sulfur protein (p-ISP). A mitochondrial intermediate peptidase (MIP) then removes an octapeptide from i-ISP to generate mature length iron-sulfur protein (m-ISP).

Intermediate iron-sulfur protein (i-ISP). A mitochondrial intermediate peptidase (MIP) then removes an octapeptide from i-ISP to generate mature length iron-sulfur protein (m-ISP). Previous reports have shown that these proteases, when extracted from mitochondria, are inhibited by metal chelators EDTA and o-phenanthroline (5–7). In _in vitro_ import studies with isolated mitochondria, we have also shown that MPP and MIP are inhibited by different concentrations of these chelators (2–4). In the present study we show that one of the main effects of high concentrations of EDTA and o-phenanthroline is the inhibition of import itself. At the concentrations where MPP in yeast mitochondria is supposedly inhibited, no import of precursor protein beyond the inner mitochondrial membrane occurs. We also show that import into the matrix space and processing of the precursor to the intermediate are two independent steps in the _in vitro_ import process.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents for _in vitro_ transcription and translation of proteins were from Promega. The _in vitro_ translation product was labeled using Tran35S-label from ICN. EDTA was from Fisher, o-phenanthroline from Sigma, m-phenanthroline from Aldrich, and p-phenanthroline from ICN.

**Isolation of Mitochondria**—Yeast strain W303-1A was grown in YPGal (1% yeast extract, 2% peptone, 2% galactose) medium to an optical density at 600 nm of 2–4. Mitochondria were isolated and frozen as described (8). 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, 100 mM KHCO3, and 0.1% bovine serum albumin, 0.5 mM Mg acetate was added and mitochondria were resuspended by centrifugation for 5 min at 16,000 × _g_ at 4°C. They were then resuspended in an appropriate volume of the same buffer.

**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) 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 MgCl2, 1 mM ATP, 4 mM NADH, 0.5 mg of mitochondrial protein, and metal chelators as indicated in a total volume of 0.8 ml. 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, m-phenanthroline, and p-phenanthroline to penetrate the mitochondrial membranes. To obtain de-energized mitochondria, a sample was withdrawn before addition of precursor and incubated for 5 min on ice in the presence of 22 μM antimycin, 20 μg/ml valinomycin, and 20 μg/ml carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone. Precursor was added to the samples and import was performed for 20 min at 30°C, while the de-energized mitochondria sample was kept on ice. Import was stopped by placing the samples on ice and adding antimycin A, valinomycin, and carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone to the concentrations indicated above.

**Fractionation of Mitochondria**—Before fractionation of the mitochondria, an 0.1-ml "mitochondria" sample was withdrawn from the import mixture and kept on ice. The remaining mitochondria were pelleted, resuspended in 2.6 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 resuspended in 0.3 ml of 0.6 M sorbitol, 3 mM MgATP, 20 mM Hepes, pH 7.4. A 50-μl "mitoplast" sample was withdrawn and kept...
on ice. The remaining mitoplasts were then disrupted with an Ultra-
sonic, sonicator for 5 × 10 s on output setting 1 and 50% duty with-
cooling on ice for 20 s between cycles. Intact mitoplasts were removed by
centrifuging for 4 min at 16,000 × g, and 0.2 ml of the supernatant was
centrifuged at 160,000 × g for 30 min. An 0.1-ml aliquot of the resulting
supernatant was withdrawn, comprising the “matrix.” The pellet was
suspending in 0.2 ml of water and 0.1 ml was withdrawn as the “mem-
brane” sample.

All samples were then divided in half and while one-half was kept on
ice, the other half was incubated with 0.1 mg/ml protease K for 30 min
on ice. Digestion was stopped by addition of phenylmethylsulfonyl flu-
oride to 2 mM and incubation on ice for 5 min. Mitochondria samples
were then pelleted and resuspended in 50 μl of water. The samples were
then analyzed by SDS-polyacrylamide gel electrophoresis and fluorog-
raphy of the dried gels.

In Vitro Transcription and Translation—In vitro transcription using
phage SP6 RNA polymerase and in vitro translation using rabbit reti-
culocyte lysate were performed according to the supplier recommend-
dations. Before use, polyomers were removed by centrifugation at
160,000 × g for 40 min. The DNA template was the pGEM-3 plasmid,
carrying wild-type S. cerevisiae RIP1 DNA (pGEM3-RIP1), or the plas-
mids pJN002, pJN003, and pJN004, each cut with ScaI.

Construction of Plasmids—pJN002, pJN003, and pJN004 were ob-
tained from pGEM3-RIP1 by changing the −10 arginine in the RIP1
presequence into alanine, lysine, or glycine, respectively, with the Clon-
tech Transformer Mutagenesis Kit.

RESULTS

EDTA and High Concentrations of o-Phenanthroline Block
Import of the Rieske Iron-Sulfur Protein into Yeast Mitochon-
dria—Import of the Rieske iron-sulfur protein and two-step
processing of the precursor protein to mature size was first
demonstrated and characterized by Hartl and co-workers (1) with
N. crassa mitochondria. Import and two-step processing of the
iron-sulfur protein to mature size was also demonstrated in S.
cerevisiae mitochondria, and the effects of different concen-
trations of EDTA and o-phenanthroline on these processes
were characterized (2–4). A combination of 10 mM EDTA and 2
mM o-phenanthroline was shown to inhibit MIP, the protease
that is responsible for the second processing step. This leads to
accumulation of i-ISP in the mitochondria.

It was also found that increasing the concentration of o-
phenanthroline to 8 mM results in accumulation of precursor
iron-sulfur protein (p-ISP), and it was proposed that this accu-
mulation results from inhibition of the MPP responsible for the
first processing step (2). However, it has never been established
that under these conditions import of the protein into the
matrix space takes place.

To determine whether import of iron-sulfur protein into the
matrix space can take place without processing of the precur-
sor, we imported the iron-sulfur protein into yeast mitochondria
in the presence of EDTA and increasing concentrations of
o-phenanthroline and then fractionated the mitochondria into
mitoplasts, inner membrane, and matrix fractions as shown in
Fig. 1. The results shown in the top panel are controls for the
subsequent conditions and are essentially identical to those
previously demonstrated with N. crassa (1) and S. cerevisiae
mitochondria (2). In the absence of metal chelators p-ISP binds
to the mitochondrial membrane but remains in a protease
accessible location if the mitochondria are de-energized with
valinomycin. If the mitochondria are import competent, the
protein is processed to mature size in two steps, and both i-ISP
and m-ISP enter a protease protected location. When the
mitochondria are subfractionated i-ISP and m-ISP are found with
both matrix and membrane fractions, where they are suscep-
tible to protease degradation.

If 10 mM EDTA is present during the incubation, as shown in
the second panel of Fig. 1, import is slowed considerably, as can
be seen by the lower amount of mature protein being formed.
The EDTA does not seem to completely inhibit either of the
proteases, although there is a slight inhibition of the MIP, as
judged by the ratio of m-ISP to i-ISP. Addition of 2.5 mM
o-phenanthroline in the presence of EDTA inhibits the second
protease step catalyzed by MIP and results in accumulation of
i-ISP, but import of the protein into the matrix space still
occurs. Increasing the o-phenanthroline concentration to 4 mM
significantly reduces the amount of protein that reaches the
matrix. At 8 mM o-phenanthroline import of protein into the
matrix space seems to be blocked, as neither form of iron-sulfur
protein can be detected in that fraction (bottom panel, Fig. 1).
The decrease in amount of iron-sulfur protein in the matrix
is paralleled by a decrease in processing of p-ISP to i-ISP, reflect-
ive of MPP activity.

Mutations in the Presequence Block Processing of Iron-Sulfur
Protein—These findings can be explained in two different
ways. One possible explanation is that import into the matrix
space is coupled to the first processing step and can only take
place after the first part of the presequence has been removed.
The second possibility is that high concentrations of o-phenan-
throline inhibit import itself, independent of any effect of the
inhibitor on MPP. This would mean that the protein is not
accepted into the mitochondrial recognition site. Since the cleavage site for MPP is usually
indicated by an arginyl residue at position −2 relative to the
cleavage site (9–11), which is position −10 respective to the
amino terminus of the mature protein, we replaced the
−10 arginine by lysine, alanine, or glycine as indicated in Fig. 2. All
of these mutations inhibit in vitro processing of a fusion protein
between the amino-terminal portion of the precursor of S.
cerevisiae cytochrome b5 and the mouse dihydrofolate reduc-
tase protein in mitochondria of S. cerevisiae and N. crassa (12).
As shown in Fig. 3, when the −10 Arg is converted to Lys, the
mutant ISP is processed to i-ISP and m-ISP similar to the
wild-type protein. One noticeable effect of the −10 Arg → Lys

![Graph](image-url)
Addition of 10 mM EDTA and increasing amounts of o-phenanthroline leads to a progressive block of import of the precursor protein into the matrix. At 8 mM o-phenanthroline a small amount of precursor is protected from proteinase K digestion in intact mitochondria. This was previously observed and interpreted as indicating that p-ISP can be imported in the absence of processing (2, 4). However, as shown by the results in Fig. 4, when the outer mitochondrial membrane is disrupted by osmotic shock, no protease protected protein can be detected in the mitoplast sample when processing is inhibited by 8 mM o-phenanthroline. This indicates that although a small amount of protein is transported beyond the outer mitochondrial membrane, no import into the matrix space takes place. Therefore the primary effect of high concentrations of o-phenanthroline is not to block the processing of precursor protein by MPP, but to inhibit import into the matrix space where the first processing step takes place. Import of the −10 Arg → Gly mutant in the absence of inhibitors (top panel, Fig. 4) also shows that import into the matrix is independent of processing of the precursor form.

**The Effect of o-Phenanthroline Is Independent of Its Ability to Chelate Divalent Cations**—To assess whether the effect of high concentrations of o-phenanthroline was dependent on its ability to chelate metals, we tested the effects of the non-chelating structural isomers m-phenanthroline and p-phenanthroline on import and processing of iron-sulfur protein. When import was performed in the presence of 10 mM EDTA and 2.5 mM m-phenanthroline or p-phenanthroline, there was no significant difference compared to import performed in the presence of 10 mM EDTA alone (Fig. 1, second panel, and Fig. 5, first and third panels). As expected for the non-chelating isomers, the second protease was not inhibited and maturation of the preprotein occurred normally. When the concentration of m- or p-phenanthroline was raised to 8 mM, however, only trace amounts of p-, i-, and m-ISP could be detected in the matrix. This indicates that import is strongly inhibited by this concentration of either m- or p-phenanthroline, despite the non-chelating nature of the compounds. Therefore, it seems likely that, in addition to metal chelation, o-phenanthroline has a secondary effect that inhibits protein import.

The inhibitory effect of high concentrations of phenanthrolines could not be attributed to uncoupling of the mitochondria. In polarographic measurements with freshly isolated yeast mitochondria 8 mM o-phenanthroline inhibited respiration and the inhibited rate was not stimulated by ADP. This effect is apparently due to chelation of a metal by the o-phenanthroline. Under these same conditions 8 mM m-phenanthroline did not inhibit respiration nor uncouple the mitochondria, since ADP stimulated respiration in the presence of the m-phenanthroline (results not shown).
Import of Yeast Rieske Iron-Sulfur Protein

of draitwas shown that when a mutation of the 2full-length precursor iron-sulfur protein.

...tional active iron-sulfur protein requires insertion of an iron-sulfur cluster, which may be subject to inhibition by chelators, and earlier results of ours indicated that mutations which blocked formation of the iron-sulfur cluster by eliminating one or more of the protoliegands also retarded, although did not fully block, maturation of the protein (14, 15).

It was earlier shown that 2 mM o-phenanthroline inhibits MIP. Our results agree with the previous findings (Fig. 1) and add to those by showing that concentrations of o-phenanthroline (2.5 mM) which block MIP processing of wild-type p-ISP do not inhibit import of p-ISP in which processing is blocked by the −10 Arg → Gly mutation (Fig. 4). However, we also found that whereas low concentrations of m-phenanthroline and p-phenanthroline have effects on import and processing different than o-phenanthroline, at high concentrations they inhibit import and processing similar to o-phenanthroline. At 2.5 mM the two non-chelating isomers allow processing of p-ISP and formation of i-ISP and m-ISP in a protease protected matrix location (Fig. 5). However, at 8 mM the non-chelating phenanthrolines cause p-ISP to accumulate in a protease accessible location in mitochondria, which indirectly blocks processing by preventing access of the matrix localized MPP to the p-ISP.

From these results we conclude that the inhibition which is seen with 8 mM o-phenanthroline is probably unrelated to its chelator activity and that at high concentrations the phenanthrolines directly inhibit a step in the import process other than proteolysis of the presequence.

REFERENCES


Fig. 5. Effects of m-phenanthroline and p-phenanthroline on import of the iron-sulfur protein. Iron-sulfur protein was imported into mitochondria in the presence of the indicated amounts of m-phenanthroline (m-Phe) or p-phenanthroline (p-Phe). The mitochondria were then fractionated and treated with proteinase K as in the legend to Fig. 1.

DISCUSSION

In the present study we have investigated whether import of the nuclear encoded Rieske iron-sulfur protein into mitochondria is obligatorily coupled to two-step processing of the precursor to mature protein by the mitochondrial presequences MPP and MIP. Previous results related to this question have been contradictory. With both N. crassa and S. cerevisiae mitochondria it was shown that when a mutation of the −10 Arg to Lys, Ala, or Gly is introduced into the presequence of the precursor of S. cerevisiae cytochrome b2 fused to mouse dihydrofolate reductase the protein is imported and not processed (12). It was thus concluded that this mutation inhibits processing but not import. In contrast to this result, a −10 Arg → Gly mutation in the presequence of pre-ornithine transcarbamylase blocked both import and processing of that protein into rat liver mitochondria, whereas mutation of the −10 Arg to Lys, Ala, or Asn allowed import and processing (13).

Our results (Fig. 3) clearly show that substitution of the −10 arginine with either lysine or alanine permits processing by both MPP and MIP. These results establish that a significant amount of p-ISP is present in a protease protected location in mitochondria under conditions where processing to i-ISP and m-ISP is occurring, although with a diminished rate which allows detectable amounts of p-ISP to accumulate. The difference between our findings and those previously described in yeast may be due to differences in the specificity of the processing presequences for the presequences of iron-sulfur protein and cytochrome b2, or may be attributable to an effect of the dihydrofolate reductase fusion protein which is not seen with the full-length precursor iron-sulfur protein.

In our experiments, of the three mutations tested, only the −10 Arg → Gly mutation completely blocked processing of p-ISP, and under these conditions significant amounts of p-ISP were imported into a protease protected location in yeast mitochondria, which was shown to be in the matrix fraction by subfractionation following import (Fig. 4). The fact that single substitutions of the equivalent arginine in the presequence of ornithine transcarbamylase blocked both import and processing may be an effect on local secondary structure as suggested (13), but which must be specific to folding of this protein or its recognition by rat liver mitochondria.

We also investigated how o-phenanthroline inhibits import of the Rieske iron-sulfur protein, following reports that at higher concentrations than used to inhibit MIP, this chelator inhibits by blocking the MPP processing. How chelators inhibit formation of m-ISP was of special interest since formation of functionally active iron-sulfur protein requires insertion of an iron-sulfur cluster, which may be subject to inhibition by chelators, and earlier results of ours indicated that mutations which blocked formation of the iron-sulfur cluster by eliminating one or more of the protoliegands also retarded, although did not fully block, maturation of the protein (14, 15).