

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*₁ 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*₁ 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)¹ first removes a 22-amino acid peptide from the presequence of the precursor iron-sulfur protein (p-ISP) to form

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 *ortho*-phenanthroline (5–7). *In vitro* import studies with yeast mitochondria have also suggested 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.

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 YP-Gal (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, 0.1% bovine serum albumin, 0.5 mM Mg 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.

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*-trifluoromethoxyphenylhydrazone. 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*-trifluoromethoxyphenylhydrazone 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 reisolated and 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

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¹ The abbreviations used are: MPP, matrix processing peptidase; MIP, mitochondrial intermediate peptidase; p-ISP, precursor iron-sulfur protein; i-ISP, intermediate iron-sulfur protein; m-ISP, mature iron-sulfur protein; Mops, 4-morpholinepropanesulfonic acid.

on ice. The remaining mitoplasts were then disrupted with an Ultrasonics, Inc. 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 $16,000 \times g$, and 0.2 ml of the supernatant was centrifuged at $160,000 \times g$ for 30 min. An 0.1-ml aliquot of the resulting supernatant was withdrawn, comprising the "matrix." The pellet was suspended in 0.2 ml of water and 0.1 ml was withdrawn as the "membrane" 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 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. Mitochondria samples were then pelleted and resuspended in 50 μ l of water. The samples were then analyzed by SDS-polyacrylamide gel electrophoresis and fluorography of the dried gels.

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 the supplier recommendations. Before use, polysomes were removed by centrifugation at $160,000 \times g$ for 40 min. The DNA template was the pGEM-3 plasmid, carrying wild-type *S. cerevisiae* RIP1 DNA (pGEM3-RIP1), or the plasmids pJN002, pJN003, and pJN004, each cut with *Sca*I.

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

RESULTS

EDTA and High Concentrations of *o*-Phenanthroline Block Import of the Rieske Iron-Sulfur Protein into Yeast Mitochondria—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 concentrations 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 accumulation 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 precursor, 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 susceptible 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

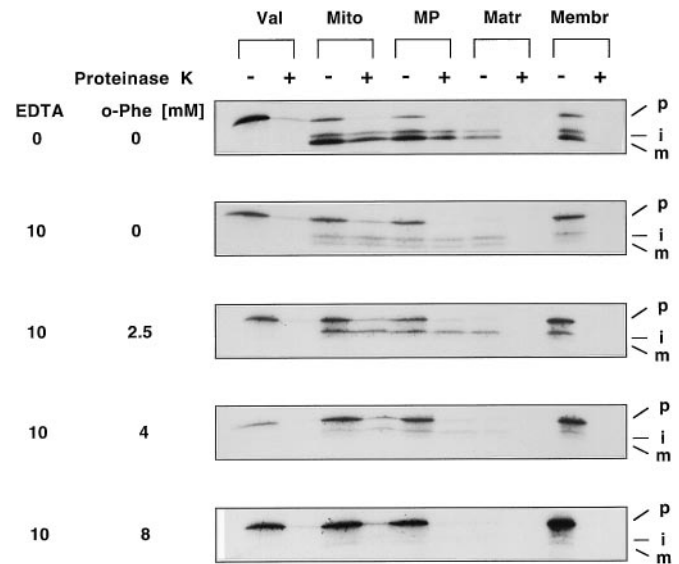


FIG. 1. Import of iron-sulfur protein in the presence of various amounts of metal chelators. Iron-sulfur protein was imported into yeast mitochondria in the presence of the amounts of metal chelators indicated in the left column. Following import the mitochondria (Mito) were fractionated into mitoplasts (MP), matrix (Matr), and membrane (Membr) fractions, which were treated with proteinase K to determine accessibility of the labeled precursor (p), intermediate (i), and mature (m) forms of the labeled proteins. (Val), mitochondria were de-energized with valinomycin, antimycin, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and kept on ice before addition of precursor.

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, reflective 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*-phenanthroline inhibit import itself, independent of any effect of the inhibitor on MPP. This would mean that the protein is not cleaved because it does not reach the compartment where processing takes place.

To distinguish between these two possibilities, we mutagenized the presequence of the iron-sulfur protein in order to inhibit the first processing step by destroying the protease 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 relative 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 *b*₂ and the mouse dihydrofolate reductase 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

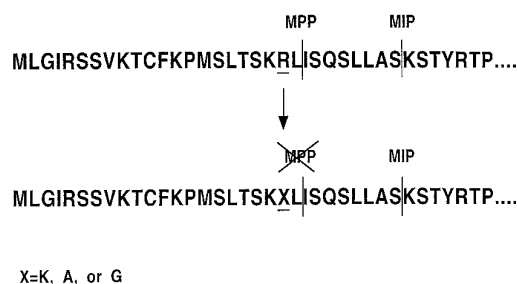


FIG. 2. **Mutational changes to the presequence of the iron-sulfur protein.** The processing sites for MPP and MIP are indicated. The -10 Arg at the MPP recognition site was changed to Lys, Ala, or Gly by site-directed mutagenesis.

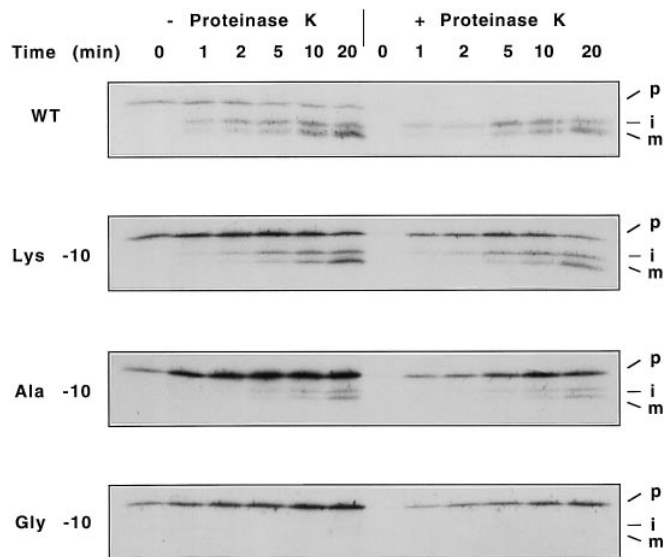


FIG. 3. **Time course of import of iron-sulfur protein mutants into mitochondria.** Wild-type iron-sulfur protein (WT) and iron-sulfur proteins in which the -10 Arg was mutated to Lys, Ala, or Gly were imported into mitochondria for the indicated times. After import half of each sample was treated with proteinase K. The migration positions of precursor (p), intermediate (i), and mature (m) iron-sulfur protein are indicated.

mutation is that the kinetics of processing appear to be altered such that a significant amount of p-ISP is protected from proteinase K, in contrast to the wild-type protein. In the -10 Arg → Ala mutant processing is slowed considerably, and in the -10 Arg → Gly mutant processing of p-ISP to i-ISP and m-ISP is almost completely blocked (*bottom panel*, Fig. 3), although upon longer exposures of these blots a very small amount of m-ISP could be detected (results not shown). The mutations do not cause the protein to become protease resistant, since complete degradation of the protein was observed in the presence of 1% Triton X-100 (results not shown).

Mutant Iron-Sulfur Protein That Is not Processed Can be Efficiently Imported into the Matrix Space—Import of the -10 Arg → Gly mutant iron-sulfur protein and subsequent fractionation of mitochondria is shown in Fig. 4. In the absence of metal chelators, a considerable amount of precursor is protected from digestion by proteinase K in mitoplasts, indicating that p-ISP has been transported beyond the outer side of the inner mitochondrial membrane. Almost all of the protected p-ISP is recovered in the matrix.

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

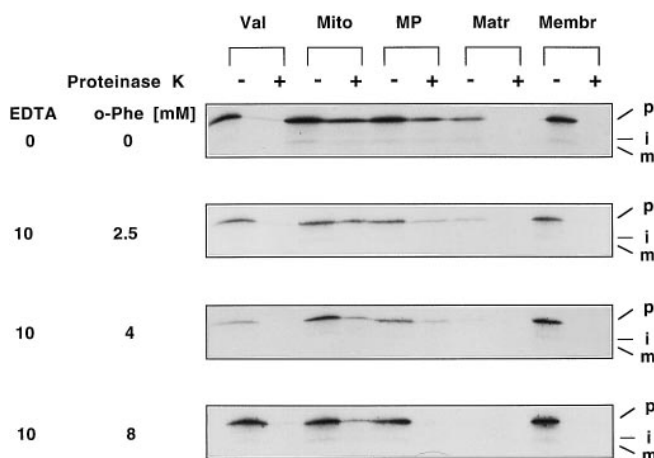


FIG. 4. **Import of the -10 Arg → Gly mutant iron-sulfur protein into mitochondria in the presence of various amounts of metal chelators.** The mitochondria were then fractionated and treated with proteinase K as in Fig. 1.

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).

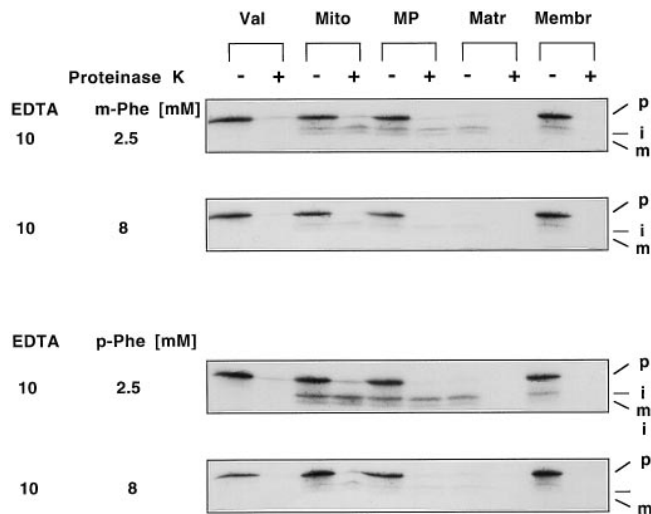


FIG. 5. Effects of *m*-phenanthroline and *p*-phenanthroline on import of the iron-sulfur protein. Iron-sulfur protein was imported into mitochondria in the presence of the indicated amounts of *m*-phenanthroline (*m*-Phe) or *p*-phenanthroline (*p*-Phe). The mitochondria were then fractionated and treated 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 proteases 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 *b*₂ 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 \rightarrow 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 proteases for the presequences of iron-sulfur protein and cytochrome *b*₂, 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 \rightarrow 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 mi-

tochondria, 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 protein ligands 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 \rightarrow 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.

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