Protein Import into Mitochondria in a Homologous Yeast In Vitro System*  

(Received for publication, October 31, 1988)  

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To study the import of proteins into mitochondria we developed a homologous in vitro system in which mitochondria and cell-free translation extract are both derived from the yeast Saccharomyces cerevisiae. This system allows the synthesis of precursor proteins in the presence of isolated mitochondria and offers a means of analyzing yeast mutants defective in mitochondrial protein import. The in vitro import of an artificial precursor protein into yeast mitochondria in the presence of its substrate analog was analyzed subsequent to synthesis in either a yeast or rabbit reticulocyte cell-free translation reaction. Results suggest that a component(s) present in the yeast cytosolic extract may interact with the precursor protein.

In the cell, the synthesis of mitochondrial precursor proteins and the initial events in the translocation of these proteins into mitochondria occur simultaneously in the cytoplasmic compartment. Therefore, to mimic the in vivo import process as closely as possible in an in vitro system, two requirements must be met. First, import must be performed in the presence of a cytoplasmic fraction derived from the same type of cell as the mitochondria. Such a system would increase the chances that any cytoplasmic factors that may play a role in the import process will be present and capable of interacting with the mitochondria. Second, import must be performed under conditions allowing protein synthesis. This requirement would reflect the in vivo situation to the extent that protein import into mitochondria and translation can operate simultaneously and might even be coupled (1–3). We chose the yeast Saccharomyces cerevisiae to develop such a homologous import system.

MATERIALS AND METHODS

 Yeast Strains and Plasmids—Mitochondrial import studies were prepared from the S. cerevisiae strain D273-10B (ATCC 26657). For the preparation of the yeast cell-free translation extract, the vacuole protease-deficient S. cerevisiae strain ABYS-1 (a) (MATa pral prb1 prc1 cpsl ade) was used. The plasmids used for in vitro transcription and translation were pDS5/2-1-pCOXIV(1–12)-DHFR, pDS5/2-1-pCOXIV(1–12)-DHFR, and pDS5/2-1-pCOXIV(1–12)-DHFR (5, 6). Plasmid pGR207, encoding the precursor form of the β-subunit of the yeast mitochondrial F1-ATPase under control of the T7 promoter, was a kind gift from Dr. Graeme Reid of the University of Edinburgh.

 Preparation of Yeast Cell-free Translation Extract—A yeast S-100 extract (7, 8) was prepared from strain ABYS-1. Cell cultures were grown in 5 liters of YPD medium (1% yeast extract, 2% Bactopeptone, 2% glucose) to an optical density of 1 at 600 nm. The cells were harvested and washed twice with water. Ten ml of packed cells were resuspended in an equal volume of lysis buffer (20 mM HEPES-KOH, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 1 mM mannitol). To the cell suspension, dithiothreitol was added to 2 mM and phenylmethylsulfonyl fluoride (PMSF) to 0.5 mM. The suspension was transferred to an AMINCO French pressure cell and frozen for 30 min in a mixture of dry ice and ethanol. Cells were lysed by applying 1600 psi hydraulic pressure. The homogenate was thawed on ice and PMSF was added again to a concentration of 0.5 mM. The homogenate was transferred to a 30-ml glass centrifuge tube and centrifuged for 10 min at 9,000 rpm in a Sorvall HB-4 rotor at 4 °C. The supernatant was collected through the upper lipid layer, avoiding the loose pellet, and centrifuged for 30 min at 100,000 × g in a Beckman 50TI rotor at 4 °C. The yellowish supernatant was collected.

 In Vitro Transcription and Translation—Capped mRNA was synthesized from pDS5/2-derived plasmids essentially as described (6) and immediately used for cell-free translation. Cell-free translation was adapted from Rothbiart and Meyer (7). A translation reaction (final volume 25 μl) contained 7 μl of transcription mixture and 10 μl of nuclease-treated yeast lysate and was adjusted to the following final concentrations: 54 mM HEPES-KOH, pH 7.4, 196 mM potassium acetate, 20 mM creatine phosphate, 80 μg/ml creatine phosphokinase, 30 μM of each amino acid except methionine, 1.1 mM ATP, 55 μM GTP, 0.2 mg/ml yeast RNA, 40 units/ml RNase inhibitor, and 10 nCi of [35S]methionine. Incubation was for 30 min at 25 °C.

 Simultaneous Cell-free Translation and Import into Mitochondria—Yeast mitochondria were prepared as described (9), except that the mitochondria were washed in breaking buffer (0.6 M mannitol, 20 mM HEPES-KOH, pH 7.4) containing 10 mM EDTA and bovine serum albumin was omitted from the preparation. A translation/import reaction with a final volume of 0.2 ml contained 70 μl (0.7 mg of protein) of yeast lysate, 0.3 mg of mitochondria, 50 μl of transcription mixture and was adjusted to the following final concentrations: 18 mM HEPES-KOH, pH 7.4, 170 mM potassium acetate, 17 mM creatine phosphate, 70 μg/ml creatine phosphokinase, 25 μM of each amino acid except methionine, 1.1 mM ATP, 50 μM GTP, 35 units/ml RNase inhibitor, 0.2 mg/ml yeast RNA, 70 μCi of [35S]methionine, and 0.1 mg/ml chloramphenicol (to block intramitochondrial protein synthesis). Incubation was for 30 min at 25 °C. In order to determine the amount of labeled protein imported into mitochondria, aliquots (50 μl) were diluted with 0.1 ml of breaking buffer, unlabeled methionine was adjusted to 3 mM, and 0.1 mg of valinomycin-treated carrier mitochondrial was added. After protease treatment (250 μg/ml...
proteins synthesized in the yeast lysate was performed essentially like the simultaneous translation/import reaction, except that mitochondria, together with 3 mM unlabeled methionine were added after the completion of the translation reaction.

Miscellaneous—Published procedures were used for cell-free translation in a rabbit reticulocyte lysate (10) and for SDS-PAGE and fluorography (11). Protein was measured by the BCA procedure as described in a company brochure ("Previews") of Pierce Chemical Co. Fluorographs were scanned using a Camag TLC Scanner II and a Camag SP4290 integrator.

RESULTS

Simultaneous Precursor Synthesis and Import into Mitochondria—Preliminary experiments demonstrated that a fusion protein consisting of the first 12 residues of the cytochrome oxidase subunit IV complex fused to mouse dihydrofolate reductase (the COXIV(1-12)-DHFR fusion protein) could be synthesized in a nuclease pretreated yeast lysate prepared by the method of Rothblatt and Meyer (7). In order to allow import to occur during the translation reaction, we added isolated mitochondria directly to the yeast lysate before the translation reaction was initiated. To do this, we had to prepare the lysate in 1 M mannitol (in place of glycerol) to make the final osmolarity of the translation/import reaction compatible with mitochondrial integrity (9). Chloramphenicol was also included to inhibit mitochondrial protein synthesis. The import reaction was started by the addition of capped mRNA derived from in vitro transcription of plasmid DNA encoding the COXIV(1-12)-DHFR fusion protein. After incubation for 30 min at 25 °C, the reaction was terminated by transferring it to ice. Further incorporation of [35S]methionine into protein was also inhibited by adding unlabeled methionine to 3 mM. When an aliquot of the reaction was analyzed by SDS-PAGE and fluorography, a single major radiolabeled protein was observed (Fig. 1, lane 1). This protein co-migrated with COXIV(1-12)-DHFR synthesized in a reticulocyte lysate and was not synthesized if mRNA encoding the fusion protein was omitted from the reaction (not shown). After sedimentation of the reaction through a sucrose cushion, approximately 20% of the fusion protein was found to be associated with the mitochondrial pellet (lane 2). Import into the organelle was confirmed by resistance to externally added proteinase K (lane 3) unless detergent was added to disrupt the mitochondrial membranes (lane 4). Approximately 15% of the protein synthesized was imported into mitochondria.

If mitochondria were uncoupled by addition of valinomycin, the fusion protein was synthesized (Fig. 1, lane 5) but was neither imported (lane 7) nor bound by the mitochondria (lane 6). This indicates that not only import, but even binding of the precursor to mitochondria in this system is potential dependent.

In order to test whether a functional mitochondrial presequence was required for import in this system, we compared the import of the precursor containing the first 12 residues of the subunit IV presequence to one containing only the first 7 residues of the presequence. The 7-residue fusion protein is non-importable in a heterologous in vitro system (5). The construct containing the functional mitochondrial presequence was imported (Fig. 2, lanes 1-4) whereas that containing the non-functional presequence (lane 5) was neither imported (lane 7) nor bound by mitochondria (lane 6). Import in the homologous translation/import system is thus both potential- and presequence-dependent.

The COXIV(1-12)-DHFR fusion protein does not contain a cleavage site recognized by the mitochondrial matrix protease (5). Therefore, to determine whether correct processing of precursor proteins occurs in the yeast homologous system, a fusion protein consisting of the first 22 residues of the cytochrome oxidase subunit IV presequence fused to DHFR and the β-subunit of the mitochondrial F1-ATPase were tested in the simultaneous translation/import reaction. Both of these precursor proteins have a cleavable presequence. When an aliquot of the reaction containing the fusion protein was analyzed by SDS-PAGE and fluorography, both the precursor and processed form were observed (Fig. 3, lower panel, lane 1). Upon sedimentation through a sucrose cushion, primarily the processed form was recovered with the mitochondrial pellet (lane 2) and was shown to be imported by its resistance to externally added protease (lane 3). Similar results were obtained with the β-subunit of the F-ATPase (Fig. 3, upper panel).

![Fig. 1. Simultaneous translation and import in the yeast homologous system. The COXIV(1-12)-DHFR fusion protein was synthesized in a yeast lysate to which 0.3 mg of yeast mitochondria per 0.2-ml final volume had been added. Translation/import was carried out for 30 min at 25 °C in the presence of 0.1 mg/ml chloramphenicol, either in the presence or absence of 10 μg/ml valinomycin (Val). Aliquots (50 μl) were diluted into breaking buffer containing 3 mM unlabeled methionine. Where indicated, mitochondria were treated with 250 μg/ml proteinase K before resolation. Protease treatment in the presence of 0.5% Triton X-100 was performed on the reisolated mitochondria where indicated. Synthesis and import were analyzed by SDS-15% polyacrylamide gel electrophoresis and fluorography. Lanes 1 and 5 are 40% of the total reaction material analyzed in lanes 2-4 and 6-7, respectively. Arrow indicates position of the precursor protein.](image-url)

![Fig. 2. A functional mitochondrial presequence is required for import in the yeast homologous system. The COXIV(1-12)-DHFR fusion protein and the COXIV(1-7)-DHFR fusion protein were synthesized in a yeast lysate to which 0.3 mg of yeast mitochondria/0.2-ml final volume had been added. The reaction was incubated for 30 min at 25 °C in the presence of 0.1 mg/ml chloramphenicol. Aliquots (50 μl) were diluted into breaking buffer ("Materials and Methods") containing unlabeled methionine. As indicated below, some aliquots were treated with 250 μg/ml proteinase K before resolation of the mitochondria, or with proteinase K and 0.5% Triton X-100 after resolation. Digestion was stopped by the addition of 1 mM PMSF. Synthesis and import were analyzed by SDS-15% polyacrylamide gel electrophoresis and fluorography. Lane 1, 40% of the total COXIV(1-12)-DHFR synthesized in the presence of the mitochondria analyzed in lanes 2-4; lane 2, reisolated mitochondria; lane 3, reisolated mitochondria treated with proteinase K; lane 4, reisolated mitochondria treated with proteinase K and Triton X-100; lane 5, 40% of the total COXIV(1-7)-DHFR synthesized in the presence of the mitochondria analyzed in lanes 6-7; lane 6, reisolated mitochondria; lane 7, reisolated mitochondria treated with proteinase K.](image-url)
FIG. 3. Import and processing of COXIV(1-22)-DHFR and the β-subunit of mitochondrial F₁-ATPase. Import of COXIV(1-22)-DHFR (lower panel) and the F₁-ATPase β-subunit (upper panel) was performed as described in the legend to Fig. 1. Lane 1, 40% of the total precursor protein synthesized in the presence of the mitochondria analyzed in the panel; lane 2, reisolated mitochondria; lane 3, reisolated mitochondria treated with proteinase K. P, precursor form; M, mature form. 22-mer, COXIV(1-22)-DHFR fusion protein.

FIG. 4. Inhibition of binding and import by methotrexate. The COXIV(1-12)-DHFR fusion protein was synthesized either in a reticulocyte or yeast lysate. Translation products were incubated for 10 min at 25 °C with or without 250 nM methotrexate and then added to isolated mitochondria. Import was analyzed as described in the legend to Fig. 1. Binding is the total amount of precursor protein reisolated with de-energized mitochondria. Import is the amount of inhibition determined by densitometric scanning of fluorographs. Filled bars, yeast system; open bars, reticulocyte system.

panel, lanes 1–3). Therefore, both the fusion protein and the authentic mitochondrial precursor protein were imported and processed in this system.

Import of the DHFR-containing Fusion Protein in the Presence of Methotrexate—The import of DHFR-containing fusion proteins synthesized in reticulocyte lysates is inhibited by preincubation with the antifolate methotrexate (12). This is because the DHFR moiety folds into its enzymatically active conformation even with the mitochondrial presequence fused to its amino terminus, suggesting that the fusion protein must at least partially unfold to be imported into isolated mitochondria. We wanted to determine if the same would be true for precursors synthesized in the homologous yeast system. The COXIV(1-12)-DHFR fusion protein was therefore synthesized either in a reticulocyte lysate or a yeast lysate in the absence of mitochondria. Following termination of translation, methotrexate was added to a final concentration of 250 nM. After incubation for 10 min at 25 °C the precursors were added to yeast mitochondria and both binding and import were analyzed. The percent inhibition caused by methotrexate in both lysate systems is shown in Fig. 4. When synthesis was performed in the reticulocyte system (open bars) methotrexate inhibited binding by about 50% and import by 80%. In contrast, when synthesis was performed in the yeast lysate (filled bars) both binding and import were significantly less inhibited by methotrexate.

A trivial explanation for the difference in methotrexate sensitivity between the reticulocyte and yeast systems is that the yeast lysate binds methotrexate. However, this is not likely because when yeast lysate was added to the fusion protein that had been synthesized in the reticulocyte lysate, there was no effect on the methotrexate sensitivity of its import (not shown).

DISCUSSION

In this article we describe a yeast homologous system for the study of protein import into mitochondria. Import in this system resembles bona fide mitochondrial import (5, 13, 14) in being both potential (Fig. 1) and prerequisite dependent (Fig. 2). However, the yeast cell-free system has several advantages over the previously used in vitro import systems. First, both the mitochondrial and cytoplasmic components are derived from yeast cells. This may be of fundamental importance since interactions between cytoplasmic factors and mitochondria, either direct or mediated through an initial interaction with precursor proteins, may well be specific that they cannot occur in heterologous systems. The relative lack of methotrexate sensitivity for import of the COXIV(1-12)-DHFR fusion protein when synthesized in the yeast lysate (Fig. 4) may indicate such an interaction and suggests that either the DHFR moiety of the fusion protein does not obtain its tightly folded, enzymatically active conformation in the yeast lysate or that a yeast cytosolic component "masks" the methotrexate-binding site.

Another principal advantage of the yeast homologous import system is that protein synthesis can occur in the presence of mitochondria. The possible occurrence and characteristics of co-translational import into mitochondria can now be explored in vitro. Also, since import occurs from a homologous cytoplasmic fraction under conditions which also permit protein synthesis, mitochondrial import in this system may resemble the in vivo process much more accurately.

Finally, the yeast homologous system can be used to analyze yeast mutants defective in mitochondrial protein import. Mitochondria from such mutants often lack in vitro phenotypes if tested in the conventional heterologous systems, perhaps because only the mutant mitochondria could be examined. With the homologous system used here, mutant cytoplasmics can be analyzed in the presence of wild-type mitochondria or vice versa, thereby offering a means of identifying mutant cytoplasmic factors participating in the import process.

Acknowledgments—We wish to thank Dr. Gottfried Schatz for his advice and criticism. Also, we thank Dr. David Meyer for help in the initial phase of this work, Kitaru Suda for excellent technical assistance, and Drs. Bernie Leniere and Alison Baker for critically reading the manuscript.

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