Coupling of Cytosolic Protein Synthesis and Mitochondrial Protein Import in Yeast

EVIDENCE FOR COTRANSLATIONAL IMPORT IN VIVO*

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We have utilized a homologous cell-free mitochondrial protein import system derived from the yeast *Saccharomyces cerevisiae*, in addition to performing a series of *in vitro* experiments in yeast, to investigate the coupling between cytosolic protein synthesis and protein transport into mitochondria. We found that the import of bulk mitochondrial proteins was inhibited in both the homologous *in vitro* reaction and *in vivo* upon arrest of cytosolic protein synthesis with the addition of cycloheximide. Tight coupling of synthesis and import was also demonstrated *in vivo* for the β subunit of the mitochondrial F$_1$-ATPase. We also investigated the effect of the antifolate methotrexate on the import of a fusion protein consisting of the mitochondrial targeting signal of yeast cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase (the COXIV-DHFR fusion protein). Methotrexate has previously been shown to inhibit posttranslational import of COXIV-DHFR by preventing the DHFR moiety from unfolding. However, we found that antifolate addition had no inhibitory effect on the import of COXIV-DHFR *in vivo*, suggesting that its import into mitochondria in yeast cells occurs cotranslationally. Further, when we treated yeast with the proton ionophore carbonyl cyanide m-chlorophenylhydrazone to collapse the mitochondrial membrane potential and induce the accumulation of extramitochondrial precursor pools, we found that the ability to be imported by a strictly posttranslational mechanism upon reestablishing the membrane potential varied from one precursor to another, suggesting that cotranslational import may be mandatory for the import of some proteins *in vivo*. In summary, our findings are entirely consistent with the notion that import of proteins into yeast mitochondria occurs cotranslationally under normal conditions *in vivo*.

Nearly all mitochondrial proteins are encoded by nuclear DNA and synthesized on cytosolic ribosomes; only a handful are encoded by the mitochondrial genome. Consequently, most mitochondrial proteins must be specifically targeted to and imported into the organelle. The mechanism by which protein targeting and subsequent membrane insertion or translocation occurs has been extensively studied in recent years (1-4). At the mitochondrial surface, import receptors recognize targeting signals and membrane insertion or translocation is thus initiated. Such signals are typically located at the extreme amino terminus of mitochondrial proteins and consequently are often referred to as "presequences." In the case of proteins destined to the mitochondrial matrix, the import process is dependent on an energized mitochondrial inner membrane and ATP hydrolysis inside the matrix. ATP hydrolysis is likely required for intramitochondrial reactions involving chaperone proteins, perhaps mediating correct folding of precursor proteins in the matrix. Once imported, amino-terminal mitochondrial presequences are usually proteolytically removed to generate mature forms of the mitochondrial proteins, which may be further sorted within the organelle or assembled into multienzyme complexes before finally performing their specific functions.

While there are still many details to be elucidated concerning the events that occur at the mitochondrial surface and inside the organelle, there are also a number of unanswered questions involving events that occur before precursor proteins engage the import receptors. For example, a considerable amount of work has resulted in the suggestion that cytosolic factors may be involved in mitochondrial protein import. Such factors include a cytosolic heat shock-like protein, hs70 (5, 6), an N-ethylmaleimide-sensitive component (7), and a 28-kDa protein isolated from both rabbit reticulocyte and rat liver lysates (8, 9). This latter component can be isolated from high salt washes of isolated rat liver mitochondria as well as from cytosolic fractions which has led the authors to suggest that the role of this factor in mitochondrial protein import may resemble that of the signal recognition particle in the transport of secretory proteins into the endoplasmic reticulum (10).

The analogy between mitochondrial protein import and protein transport into the endoplasmic reticulum may be even more extensive. Evidence exists suggesting that protein translocation across both membrane systems may occur cotranslationally (11). In a cotranslational transport reaction, polypeptides are translocated across a membrane while still being synthesized. In the case of the endoplasmic reticulum, cotranslational protein translocation is usually considered as a major transport pathway, and a great deal of mechanistic detail related to the process has been reported. On the other hand, the involvement of a cotranslational import mechanism for protein transport into mitochondria is not as widely accepted. The ability to import proteins in strictly posttranslational heterologous *in vitro* reactions has demonstrated that the import process can occur subsequent to termination of precursor synthesis. Such *in vitro* systems typically consist of a cell-free protein translation system, usually derived from...
rabbit reticulocytes and mitochondria isolated from other sources, often either fungal or mammalian. However, we have recently reported that in a homologous in vitro import reaction, consisting of a cell-free translation extract and mitochondria both isolated from the yeast Saccharomyces cerevisiae, cotranslational protein import is much more efficient than posttranslational import; precursor synthesis and import are very tightly coupled (12).

While in vitro import reactions have been extremely useful in exploring the mechanism of mitochondrial protein transport, they are only approximations of the in vivo condition, and their relevance to what actually occurs in living cells must constantly be evaluated. This is particularly applicable for phenomena such as cotranslational protein import into mitochondria where two complex, multistep reactions, cytosolic protein synthesis and mitochondrial protein transport, are involved. In this report, we present data demonstrating that in yeast precursor synthesis and import are very tightly coupled in vivo and that a cotranslational import mechanism may actually be required for some precursor proteins.

MATERIALS AND METHODS

Yeast Strains—S. cerevisiae strain D273-10B (ATCC 25657) was used for time course experiments with cycloheximide as well as for isolation of yeast mitochondria for in vitro import reactions. Strain DAU1 (MATa ade ura3), harboring the plasmid, pYVR, carrying the gene for the COXIV(1-22)DHFR fusion protein (22 amino-terminal residues of the cytochrome oxidase subunit IV presequence fused to mouse dihydrofolate reductase) (13) under control of ADHI promoter (22) was utilized in this study. Yeast mitochondria were isolated as described (12). Yeast mitochondria were both derived from the yeast S. cerevisiae strain D273-10B (ATCC 25657) and that a cotranslational import mechanism is much more efficient than posttranslational import; precursor synthesis and import are very tightly coupled (12). Yeast mitochondria were adjusted to the same final concentration following protein determination of individual samples.

Yeast Cells and Spheroplasts—Spheroplasts were prepared from the wild-type yeast strain D273-10B grown to mid-log phase in a low sulfate semisynthetic medium (0.3% yeast extract, 0.04% CaCl2, 0.05% NaCl, 0.059% MgCl2·6H2O, 0.1% KH2PO4, 0.98% NH4Cl) containing 2% lactate, pH 5.5 as described (15). The spheroplasts were collected by centrifugation and resuspended in 1 g wet cells/ml in 40 mM K5P, pH 6.0, containing 0.05% yeast extract and 1% glucose. Radiolabeling of intact cells was as described above for spheroplasts. The yeast strain DAU1-YVR was incubated at 30 °C to mid-log phase in synthetic minimal medium (0.68% yeast nitrogen base, 0.05% glucose, 20 mg/liter adenine, and 2% lactate, pH 5,5), diluted with the same volume of fresh medium, and further incubated at 30 °C as indicated in the figure legends. Where indicated, sultamil (5 mg/ml) was included in the medium. Cells were then collected, resuspended to 0.1 g/ml in 40 mM K5P, pH 6.0 containing 1% glucose, and labeled as described above. Where indicated, 20 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) was included in the medium to disrupt mitochondrial membrane potential, and β-mercaptoethanol was added to 0.05% (v/v) to re-establish the membrane potential (16, 17).

Import of Proteins into Mitochondria in a Yeast Homologous in Vitro System Is Tightly Coupled to Protein Synthesis—In an earlier study, we utilized an in vitro mitochondrial protein import reaction in which the cell-free translation extract and mitochondria were both derived from the yeast S. cerevisiae (19). Using this system, we demonstrated that the import of mitochondrial precursor proteins was very tightly coupled to their synthesis; the addition of either cycloheximide or puromycin abruptly halted both precursor synthesis and import (12). In the experiment shown in Fig. 1, we examined the import of bulk proteins into mitochondria in this system. The yeast lysate in this experiment was not treated with nucleases to degrade endogenous mRNA so that we could follow the import of bulk authentic proteins into energized mitochondria. Total protein synthesis (solid lines, open squares) and protein import into mitochondria (dashed lines, open triangles) were examined both 3 and 6 min after the reaction was initiated. Another sample was incubated for 3 min, cycloheximide was added to 100 μg/ml, and the reaction was then incubated for an additional 3 min before total protein synthesis and mitochondrial import were determined (filled symbols). As can be seen, the addition of cycloheximide at 3 min immediately stops both total protein synthesis and protein import into mitochondria. While only a 3-min time point is shown in Fig. 1, no further protein synthesis or import occurred after cycloheximide addition regardless of incubation time. These results strongly suggest that precursor synthesis...
and import are very tightly coupled in the homologous in vitro import system.

Import of Proteins into Mitochondria in Vivo Is Tightly Coupled to Cytosolic Protein Synthesis—While the experiment shown in Fig. 1 suggests that precursors may be imported into mitochondria cotranslationally in the yeast homologous in vitro import reaction, we wanted to determine if such tight coupling of cytosolic protein synthesis and import also occurs in vivo. We therefore performed an experiment similar to the one shown in Fig. 1 with yeast spheroplasts. Spheroplasts were incubated in the presence of [35S]methionine at 30 °C for either 3 or 6 min, and then total cellular protein synthesis was determined (Fig. 2, solid lines, open squares). Mitochondria were then isolated from both samples to determine the amount of protein imported into the organelles at each time point (dashed lines, open triangles). To another sample, cycloheximide was added to a final concentration of 1 μg/ml at 3 min and the sample was allowed to incubate at 30 °C for an additional 3 min before total protein synthesis and protein import into mitochondria were determined (filled symbols). Finally, another sample was treated with cycloheximide from the beginning of the incubation so that the amount of [35S]methionine incorporation into protein by mitochondrial translation at both 3 min and 6 min could be determined and subtracted from total cellular protein synthesis. Protein synthesis in Fig. 2 therefore represents only cycloheximide inhibitable, or cytosolic protein synthesis. It is clear from this experiment that we obtain a remarkably similar result in vivo as when we perform the same type of experiment in the yeast homologous in vitro reaction (Fig. 1); thus when cytosolic protein synthesis is arrested by the addition of cycloheximide, protein import into mitochondria is immediately arrested as well. While this experiment was performed at 3 and 6 min incubation periods, we have obtain identical results when 2 and 3 min time points were analyzed and when incubation was continued for as long as 20 min after cycloheximide addition (data not shown). Consequently, the import of proteins into mitochondria is very tightly coupled to cytosolic protein synthesis in vivo.

The experiment illustrated in Fig. 2 depicts total acid-insoluble counts imported into mitochondria. While it demonstrates that bulk protein import into mitochondria is tightly coupled to cytosolic protein synthesis, we wished to determine if the import of a subset of mitochondrial proteins occurred subsequent to inhibition of cytosolic protein synthesis. Therefore, an aliquot of each sample of isolated mitochondria analyzed in Fig. 2 was subjected to SDS-polyacrylamide gel electrophoresis and fluorography so that we could visualize individual imported proteins (Fig. 3). Lanes 1 and 2 represent mitochondria isolated at 3 and 6 min, respectively, that were treated with cycloheximide from the beginning of the incubation and consequently represent the amount of protein translation on mitochondrial ribosomes at those times. In identical experiments in which we included chloramphenicol (an inhibitor of protein synthesis on mitochondrial ribosomes), the bands observed in lanes 1 and 2 were not present (data not shown). After 3 min of incubation, mitochondria that were isolated from spheroplasts not treated with cycloheximide imported a multitude of different proteins (lane 3). When incubation was continued in the absence of cycloheximide for an additional 3 min, the import of essentially all mitochondrial proteins increased (lane 5). On the other hand, if cycloheximide was added after 3 min of import and then incubation was continued for another 3 min, we were unable to observe an increase in the import of any mitochondrial proteins (lane 4). Consequently, while the mitochondria were capable of approximately doubling the total amount of protein imported between 3 and 6 min (see Fig. 2), this increase was entirely dependent on continued cytosolic protein synthesis for each individual mitochondrial protein we could detect.

The experiments shown in Figs. 2 and 3 depict the import of bulk authentic proteins into mitochondria in vivo. In order to confirm the tight coupling of synthesis and import of a mitochondrial protein known to be targeted into the matrix in vivo, as well as to demonstrate that this phenomenon is not limited to experiments using yeast spheroplasts, we performed a similar experiment with intact, log-phase yeast cells (Fig. 4). In this experiment, cells were incubated for either 3 or 6 min in the presence of [35S]methionine. Also, one sample was incubated for 3 min after [35S]methionine addition; cycloheximide was added to 100 μg/ml, and then the reaction was incubated for another 3 min. Each sample was then solubilized and analyzed by immunoprecipitation with antiserum directed against the β subunit of yeast mitochondrial F1-ATPase (F1β). This subunit is synthesized on cytosolic ribosomes with a cleavable amino-terminal presequence (20). Consequently, import of this mitochondrial protein can be followed in whole cell extracts by the presence of its imported “mature” form which migrates more rapidly on SDS-polyacrylamide gels (indicated as m in Fig. 4). In lane 1, the immunoprecipitation procedure was conducted on a whole cell extract obtained from cells incubated for 6 min in the presence of [35S]methionine using preimmune serum, demonstrating the immunospecificity of the F1β antiserum. Lanes 2 and 4 illustrate the import of F1β in the absence of cycloheximide for 3 and 6 min, respectively. Densitometric analysis of the fluorograph...
Mitochondria were then isolated from each sample for determination of the import of bulk mitochondrial proteins (22). As described under "Materials and Methods," the samples were analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Lane 1, immunoprecipitation with preimmune serum of a sample identical to that analyzed in lane 4; Lanes 2-5, immunoprecipitation with an antiserum against F1β. Lane 2, F1β import at 3 min; lane 3, cycloheximide added at 3 min and incubation continued for an additional 3 min; lane 4, at 6 min; lane 5, F1β incubated for 6 min in the presence of CCCP. p, precursor; m, mature-sized form of F1β.

In Vivo Import of COXIV-DHFR Is Not Inhibited by the Substrate Analog Methotrexate and Must Occur Cotranslationally—Fusion proteins consisting of the mitochondrial targeting signal of yeast cytochrome oxidase subunit IV fused to the amino terminus of the mouse cytosolic enzyme dihydrofolate reductase (COXIV-DHFR fusion proteins) have been shown to import efficiently into yeast mitochondria both in vitro and in vivo (22). A unique and very useful property of COXIV-DHFR fusion proteins is that their import into mitochondria in heterologous in vitro import reactions can be inhibited by the addition of the antifolate methotrexate (23). This substrate analog inhibits import by binding to the DHFR moiety of fusion proteins containing it and prevents their unfolding. Due largely to this useful property, COXIV-DHFR fusion proteins have been extensively utilized to study a number of mechanistic details of the import process. However, we have shown that the addition of methotrexate had very little inhibitory effect on the import of a COXIV-DHFR fusion protein in the yeast homologous in vitro import system (19). Since a protein that is imported into mitochondria as it is being translated, before its methotrexate-binding site has even been synthesized, would not be expected to be inhibited by the substrate analog, the inability of methotrexate to inhibit import in the yeast homologous in vitro reaction is consistent with the cotranslational nature of this system. As methotrexate freely enters yeast cells (24), we decided to use the drug as a means of further exploring the tight coupling between precursor synthesis and import in vivo. We therefore performed experiments to determine if methotrexate would inhibit the import of a fusion protein consisting of the first 22 residues of the subunit IV matrix targeting signal fused to mouse DHFR (Fig. 5). This COXIV-DHFR fusion protein has a cleavable presequence, thus allowing us to follow its import into mitochondria in vivo from whole cell extracts by the presence of its cleavage product. A plasmid harboring the

FIG. 4. Effect of cycloheximide on the import of F1β in vivo. Wild-type yeast cells were grown in semisynthetic medium containing 2% lactate (pH 5.5) to mid-log phase, harvested, and resuspended to 0.1 g wet cells/ml in 40 mM KPi, pH 6.0 containing 0.05% yeast extract and 1% glucose. The cells were then labeled with [35S]methionine either in the presence or absence of 20 μM CCCP at 30 °C. At 3 min an aliquot was withdrawn from the reaction containing no CCCP, and cycloheximide was added to 100 μg/ml. The aliquot was then divided into two halves, one of which was transferred to ice and the other returned to 30 °C for an additional 3 min of incubation before being transferred to ice. At 6 min the remainder of the reaction and the reaction containing CCCP were transferred to ice and cycloheximide was added to 100 μg/ml. The cells from each sample were broken and the F1β was immunoprecipitated as described under "Material and Methods." The samples were analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Lane 1, immunoprecipitation with preimmune serum of a sample identical to that analyzed in lane 4; Lanes 2-5, immunoprecipitation with an antiserum against F1β. Lane 2, F1β import at 3 min; lane 3, cycloheximide added at 3 min and incubation continued for an additional 3 min; lane 4, at 6 min; lane 5, F1β incubated for 6 min in the presence of CCCP. p, precursor; m, mature-sized form of F1β.

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FIG. 3. Import of bulk mitochondrial proteins in vivo. Mitochondrial fractions at 3 and 6 min from the experiment shown in the Fig. 2 were analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Lane 1, mitochondrial fraction at 3 min from the control reaction; lane 2, 6 min from the control reaction; lane 3, import of bulk mitochondrial proteins at 3 min; lane 4, import for 3 min and followed by an additional 3 min of incubation in the presence of cycloheximide; lane 5, import at 6 min.

shown in Fig. 4 demonstrated that the amount of imported F1β approximately doubled between the 3- and 6-min time points. On the other hand, if cycloheximide was added after 3 min of import and then incubation was continued for another 3 min, no additional F1β import is observed (lane 3). In order to confirm that lanes 2-4 represent the mature, imported form of F1β as well as to show that precursor cleavage does not occur during preparation of the samples for analysis, we

FIG. 2. Effect of cycloheximide on the import of bulk mitochondrial proteins in vivo. Wild-type yeast (D273-10B) was grown in semisynthetic medium containing 2% lactate (pH 5.5) to mid-log phase, converted to spheroplasts, and resuspended to 0.1 g wet cells/ml in 40 mM KPi, pH 6.0, containing 1.2 M Sorbitol, 0.05% yeast extract, and 1% glucose. The spheroplasts were divided into control and main reactions immediately after the addition of [35S]methionine. Cycloheximide was added to the control reaction to 100 μg/ml. Both reactions were then incubated at 30 °C. At 3 min an aliquot was withdrawn from the main reaction, and cycloheximide was added to 100 μg/ml. The sample was then divided into two halves, one of which was transferred to ice and the other returned to 30 °C for an additional 3 min before being transferred to ice. At 6 min the remainder of the reaction was transferred to ice. A 2-μl sample was taken from each aliquot to determine total protein synthesis by trichloroacetic acid-precipitation counts. Mitochondria were then isolated from each sample as described under "Materials and Methods" and treated with 250 μg/ml proteinase K for 15 min at 0 °C followed by 1 mM PMSF. A 2-μl sample was taken from the mitochondrial fractions isolated from each aliquot for determination of the import of bulk mitochondrial proteins by trichloroacetic acid-precipitable counts. Synthesis and import were also determined at 3 and 6 min from the control reaction which received cycloheximide at the beginning and were used to subtract the background. Both synthesis and import are presented as CPM × 10−5. Solid lines represent total cytosolic protein synthesis. Dashed lines represent import of bulk mitochondrial proteins.
with fresh medium containing 5 mg/ml sulfanilamide, and incubated for additional 2 h. The cells were then harvested and resuspended to 0.1 g wet cells/ml in 40 mM KPi, pH 6.0 containing 1% glucose and 5 mg/ml sulfanilamide. The cells were then divided into two samples, one of which received methotrexate to a final concentration of 0.2 mM. Both samples were then radiolabeled at 30 °C for 10 min either in the presence or absence of 20 μM CCCP. The reactions labeled for 10 min in the absence of CCCP were subjected to immunoprecipitation immediately after the addition of cycloheximide to 100 μg/ml to determine the import of COXIV-DHFR fusion protein. The reactions labeled in the presence of CCCP were chased for 10 and 20 min after the addition of cycloheximide and β-mercaptoethanol to 100 μg/ml and 0.05%, respectively. The COXIV-DHFR fusion protein was then immunoprecipitated from each sample. Immunoprecipitates were analyzed by SDS-12% polyacrylamide gel electrophoresis. Lane 1, preimmune serum control; lane 2, immunoprecipitation with an antiseraum directed against mouse DHFR; lanes 2 and 3, the fusion protein labeled in the absence and presence of 0.2 mM methotrexate, respectively; lane 4, labeled for 10 min in the presence of 20 μM CCCP; lanes 5 and 6, 10- and 20-min chase, respectively; Lanes 7–9, treated identically to lanes 4–6 except in the presence of 0.2 mM methotrexate, p, precursor; m, mature-sized form of COXIV-DHFR.

The experiments described above (Figs. 2–4) suggest that if cytosolic pools of mitochondrial precursor proteins exist, such pools must be extremely small under normal physiological conditions. Consequently, in order to study the effect of methotrexate on a strictly posttranslational import mechanism necessitated the accumulation of the COXIV-DHFR precursor in vivo. To induce this accumulation, we again made use of the finding that uncoupling mitochondria with the proton ionophore CCCP strongly inhibits protein import in vivo and leads to the accumulation of mitochondrial precursor proteins (see Fig. 4). Indeed, when CCCP was present during a 10-min pulse with [35S]methionine, only the precursor form of COXIV-DHFR was present (Fig. 5, lane 4), demonstrating the in vivo accumulation of the COXIV-DHFR precursor in the absence of a potential across the mitochondrial membrane. It has been previously shown that the effect of CCCP on the mitochondrial membrane potential in vivo can be reversed by the addition of β-mercaptoethanol (16, 17).

In order to test for posttranslational import of the accumulated COXIV-DHFR precursor, β-mercaptoethanol was added to cells that were labeled for 10 min in the presence of CCCP; cycloheximide was then added to inhibit further protein synthesis and the cells were incubated for an additional 10 or 20 min (lanes 5 and 6, respectively). Surprisingly, very little of the accumulated precursor could be chased posttranslationally into the reenergized mitochondria. The small amount of the mature COXIV-DHFR that was formed upon the addition of β-mercaptoethanol did not increase between the 10- and 20-min chase. In addition, an identical amount of the mature form was generated even if 0.2 mM methotrexate was present during the chase (lanes 7–9).

It has previously been reported that the accumulated precursor of F1β could be chased into mitochondria in vivo after β-mercaptoethanol addition to CCCP-treated yeast cells (16, 17). In order to determine if F1β could chase into mitochondria postranslationally in vivo under our experimental conditions, we performed immunoprecipitation on some of the same samples used in the experiment shown in Fig. 5 with antiserum directed against yeast F1β (Fig. 6). When cells were labeled for 10 min and immunoprecipitation was performed with preimmune serum, no F1β was found in the pellet (lane 1). On the other hand, when cells were labeled for 10 min in the presence of CCCP and then immunoprecipitation was performed with anti-DHFR antiserum, the accumulated precursor form of the protein was clearly detectable (lane 2). When β-mercaptoethanol was added to cells labeled in the presence of CCCP, followed by another 10-min incubation in the presence of cycloheximide, all of the accumulated precursor was chased into the reenergized mitochondria (lane 3), demonstrating that F1β can be imported postranslationally in vivo.

It has recently been reported that the membrane potential required to drive protein import into mitochondria may vary from one precursor protein to another (25). It is therefore possible that while the addition of β-mercaptoethanol to CCCP-treated cells reestablishes the membrane potential to an extent sufficient for the posttranslational import of F1β, this same potential is not adequate to support the import of COXIV-DHFR. Alternatively, the CCCP/β-mercaptoethanol treatment may damage mitochondria by some undefined mechanism that inhibits the posttranslational import of COXIV-DHFR more than F1β. In order to exclude these possibilities, we performed the control experiment shown in Fig. 7. When cells were labeled for 10 min in the absence of CCCP, large amounts of the imported, mature forms of the protein were detectable (lane 2). On the other hand, when CCCP was present during 10 min of labeling with [35S]methionine the precursor form of COXIV-DHFR accumulated (lane 3). As before, upon the addition of cycloheximide and β-mercaptoethanol, very little of the accumulated COXIV-DHFR protein was chased into the reenergized mitochondria after an additional 10 or 20 min of incubation (lanes 4 and 5). Finally, in another sample, cells were treated for 10 min with CCCP in the absence of [35S]methionine. β-Mercaptoethanol was then added together with [35S]methionine, and the cells were incubated for an additional 10 min before import.
Fig. 7. Cotranslational import of the COXIV-DHFR fusion protein in vivo. Yeast cells (DAV1) transformed with a plasmid carrying the gene for COXIV-DHFR were grown overnight in synthetic minimal medium containing 2% lactate (pH 5.5) to mid-log phase, diluted (1:1) with fresh medium, and incubated for additional 2 h at 30 °C. The cells were then harvested and resuspended to 0.1 g/ml in 40 mM KPi, pH 6.0 containing 1% glucose. Cells were divided into 3 aliquots. The first was labeled with [35S]methionine for 10 min and the import of COXIV-DHFR determined by immunoprecipitation from the total cell extract. The second aliquot was pulse-labeled for 10 min in the presence of 20 μM CCCP and chased for 10 and 20 min upon addition of cycloheximide and β-mercaptoethanol to 0.1 mg/ml and 0.05%, respectively. 660-μl samples were taken from each time point and subjected to immunoprecipitation. The third aliquot was incubated in the presence of 20 μM CCCP for 10 min and then labeled with [35S]methionine for 10 min upon addition of β-mercaptoethanol to 0.05%. A 660-μl sample was taken at the end of 10 min of labeling and subjected to immunoprecipitation. The immunoprecipitates were analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Lane 1, immunoprecipitation with preimmune serum of a sample identical to that analyzed in lane 2; lanes 2-6, immunoprecipitation with an antiseraum against mouse DHFR; lane 2, labeled for 10 min in the absence of CCP; lane 3, labeled for 10 min in the presence of 20 μM CCP; lane 4, 10-min chase; lane 5, 20-min chase; lane 6, labeled for 10 min after CCP/β-mercaptoethanol treatment.

COXIV-DHFR was radiolabeled only after completion of the CCCP/β-mercaptoethanol treatment. As can clearly be seen, COXIV-DHFR was efficiently imported into the CCCP/β-mercaptoethanol-treated mitochondria (lane 6). Therefore, the extreme inefficiency of posttranslational import of accumulated precursor protein (Fig. 7, lanes 4 and 5) is not due to the inability of the mitochondria to import the fusion protein; cells treated identically can efficiently import COXIV-DHFR provided that precursor import is tightly coupled to its synthesis. As the in vivo import of COXIV-DHFR is not inhibited by methotrexate (Fig. 5) and cannot be chased into mitochondria efficiently from accumulated cytosolic pools (Fig. 7), we conclude that this protein is translocated into mitochondria cotranslationally in vivo and that a cotranslational import mechanism is apparently mandatory for its import.

**DISCUSSION**

In this report we have demonstrated that the synthesis and import of mitochondrial precursor proteins are very tightly coupled both in a yeast homologous in vitro import reaction and in vivo. Further, we have demonstrated that the import of at least one mitochondrial precursor protein must occur cotranslationally; its import is mechanistically coupled to its synthesis in vivo.

That protein import into mitochondria occurs cotranslationally was originally suggested a number of years ago based on several observations obtained from yeast in vivo experiments (see Ref. 11, for review). One of the most relevant of these observations was that when mitochondria were isolated from cycloheximide-treated yeast, cytosolic polysomes were found bound specifically at points of close association between the inner and outer mitochondrial membranes (26). Such “contact sites” are now known to be the precise location on the organelle surface where protein import occurs. Also, when the mitochondrial-bound polysomes were isolated and the mRNA they contained was analyzed, it was found to be enriched for a number of mitochondrial proteins (27, 28). Yet, another series of experiments conducted at approximately the same time demonstrated that if the precursor of mitochondrial F1β was accumulated by uncoupling mitochondria in vivo with CCCP, it could be chased into mitochondria posttranslationally when the membrane potential was reestablished (16, 17). We have reproduced this experiment and find essentially the same result (Fig. 6). However, it is important to note that in these experiments in which very short pulses are performed and that the addition of cycloheximide abruptly arrests both the synthesis and import of this protein (Fig. 4). We therefore suggest that while the import of F1β can occur posttranslationally in vivo it does not normally do so. Instead, we suspect that it is imported cotranslationally into mitochondria in vivo as are most other mitochondrial proteins (Figs. 2 and 3).

Unlike the F1β protein, we found that the COXIV-DHFR precursor that was accumulated in the presence of CCCP could not be efficiently imported posttranslationally into mitochondria in vivo (Figs. 5 and 7). This is an extremely significant result for at least two reasons. First, since COXIV-DHFR is not imported efficiently into mitochondria in vivo by a strictly posttranslational import reaction, it constitutes compelling evidence that this protein is transported cotranslationally into mitochondria under normal physiological conditions. Second, in a previous study we demonstrated that COXIV-DHFR can only be imported cotranslationally in vitro when import is performed in a homologous reaction in which all of the components are derived from yeast. In this yeast homologous in vitro system, many other authentic mitochondrial proteins are imported into isolated mitochondria only when membrane transport is coupled to cytosolic protein synthesis (12). The inability of COXIV-DHFR to be imported efficiently into mitochondria posttranslationally in vivo may therefore suggest that there exists a subset of mitochondrial proteins whose import is mechanically coupled to their synthesis. If this is the case, it is possible that a specific mechanism exists to ensure that the coupling of precursor synthesis and mitochondrial import takes place, at least for some proteins. Such a mechanism may involve cytosolic components that function in a manner similar to that of yeast signal recognition particle in protein transport into the endoplasmic reticulum, which also appears to be required for only a subset of proteins in the secretory pathway (29). In any event, since COXIV-DHFR must be imported cotranslationally in vivo, it should be a useful tool for studying cellular conditions or mutations that specifically stimulate or inhibit cotranslational protein import into mitochondria.

In a recent study, the import of a fusion protein consisting of mouse DHFR fused to the amino-terminal 167 amino acid residues of yeast cytochrome b3 was found to be partially inhibited by the antifolate aminopterin (30). Like methotrexate, aminopterin likely inhibits import of fusion proteins containing DHFR by binding to the DHFR moiety and preventing protein unfolding. Antifolate inhibition of b3-DHFR import was consequently taken as evidence that a significant percent of this fusion protein was imported posttranslationally in vivo. However, in the present study we demonstrate that under essentially identical conditions the in vivo import of the COXIV-DHFR fusion protein is not inhibited by methotrexate (Fig. 5). In this respect, our results are consistent with those obtained in a recent in vivo study which demonstrated that the import of a fusion protein containing the F1β targeting sequence attached to a yeast copper-binding protein was not affected by the addition of Cu++, which was shown to inhibit its posttranslational import in vitro (31). There is not an obvious explanation for these apparently conflicting re-
Cotranslational Protein Import into Mitochondria

results. It is, however, possible that different mitochondrial targeting signals differ in their bias for a co- or posttranslational import pathway. To explore this interesting possibility, we are currently performing experiments to analyze the involvement of presequence information in directing either co- or posttranslational import both in vivo and in the yeast homologous in vitro system. In any case, our results clearly demonstrate that the import of most authentic mitochondrial proteins is very tightly coupled to their synthesis in vivo (Figs. 2 and 3).

It has been suggested that protein import into mitochondria as well as protein transport into the endoplasmic reticulum can be inhibited in vivo when cytosolic hsp70 concentrations are reduced (5). These observations have been interpreted to indicate a direct role for cytosolic hsp70 in mitochondrial protein import. At first, we considered that the limited amount of mature COXIV-DHFR generated upon reestablishment of the mitochondrial membrane potential after CCCP treatment might infer the involvement of cytosolic hsp70 in the import process (Fig. 7). That is, the accumulated COXIV-DHFR precursor may require hsp70 for posttranslational import, but so much precursor accumulates during the CCCP treatment that hsp70 becomes saturated; consequently only a fraction of the precursor is imported. Consistent with this interpretation is the finding that the apparent posttranslational import of COXIV-DHFR is not inhibited by the substrate analog methotrexate (Fig. 5). However, we find this explanation to be unlikely for a number of reasons. First, hsp70 is thought to act catalytically, yet there was no increase in posttranslational COXIV-DHFR import between the 10- and 20-min chase (Fig. 5). Second, analysis of the exact same samples revealed that F1β chased quite efficiently into mitochondria even though it is the only mitochondrial protein that has been shown to be affected by reduced cytosolic hsp70 concentrations in vivo (5). Finally, the COXIV-DHFR precursor protein has been purified following overexpression in Escherichia coli and shown to import efficiently into isolated yeast mitochondria without the addition of any cytosolic factors (23). Perhaps a more plausible explanation to account for the small fraction of accumulated COXIV-DHFR that was imported after reestablishing the membrane potential in our experiments is that it represents a small pool of an in vivo import intermediate that bound to import receptors on the mitochondrial surface as nascent precursor chains before the receptors became saturated during CCCP treatment.

Nonetheless, cytosolic hsp70 has been implicated in the in vivo import of proteins into mitochondria (5). Therefore, since our results indicate that the synthesis and import of mitochondrial proteins are tightly coupled under physiological conditions in vivo, it is possible that cytosolic hsp70 may be involved in a cotranslational import reaction. Consequently, the mechanism by which hsp70 acts in the import process may be quite different than is currently suspected: that of binding to and maintaining loosely folded conformations of soluble pools of precursor proteins. For example, hsp70 may interact with a nascent precursor chain, preventing its aminoterminal presequence from folding back into the growing peptide chain or even onto the ribosome itself before it engages a mitochondrial import receptor. Consistent with this notion is the finding that cytosolic hsp70 appears to interact cotranslationally with newly synthesized proteins and that primarily nascent chains are immunoprecipitated with hsp70 antibody under normal physiological conditions (32).

While our results demonstrate that the synthesis and import of mitochondrial proteins has a strong cotranslational component in vivo, we must emphasize that they do not exclude the possibility of posttranslational import. On the contrary, it is clear that at least the accumulated precursor of F1β can be imported efficiently in vivo (Fig. 6). In this context, it is interesting to note that while in vivo precursor pools are undetectable in some studies (33), small pools have been reported in others (16). It is therefore entirely possible that the degree to which a posttranslational mechanism participates in the in vivo import of proteins into mitochondria may vary. For example, a cotranslational mechanism likely predominates under physiological conditions where the demand for mitochondrial protein import is equal to or exceeds the rate at which the cytosolic translation machinery can synthesize precursor proteins. On the other hand, reduced import rates or accelerated cytosolic protein synthesis may result in a posttranslational import reaction. Clearly, in our experiments the contribution of posttranslational protein import into mitochondria in vivo was negligible. In any case, the finding that COXIV-DHFR cannot be imported posttranslationally in vivo (Figs. 6 and 7) is extremely significant as it suggests that cotranslational import of some precursor proteins may be mandatory. Consequently, cellular conditions that inhibit cotranslational import could, in effect, regulate the ability of some proteins to be imported. We are currently involved in experiments to identify other proteins with mandatory cotranslational import requirements to explore this exciting possibility further.

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