Import of cytochrome b_2 to the mitochondrial intermembrane space: The tightly folded heme-binding domain makes import dependent upon matrix ATP

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Abstract

Cytochrome b_2 is synthesized as a precursor in the cytoplasm and imported to the intermembrane space of yeast mitochondria. We show here that the precursor contains a tightly folded heme-binding domain and that translocation of this domain across the outer membrane requires ATP. Surprisingly, it is ATP in the mitochondrial matrix rather than external ATP that drives import of the heme-binding domain. When the folded structure of the heme-binding domain is disrupted by mutation or by urea denaturation, import and correct processing take place in ATP-depleted mitochondria. These results indicate that (1) cytochrome b_2 reaches the intermembrane space without completely crossing the inner membrane, and (2) some precursors fold outside the mitochondria but remain translocation-competent, and import of these precursors in vitro does not require ATP-dependent cytosolic chaperone proteins.

Keywords: chaperones; intermembrane space; mitochondria; protein sorting; protein translocation; stop-transfer

Most mitochondrial proteins are synthesized in the cytoplasm and must be imported across one or both organelle membranes to reach their final destinations. This import process has been analyzed extensively during the past decade (Glick & Schatz, 1991; Glover & Lindsay, 1992; Wienhues & Neupert, 1992). Mitochondrial targeting signals are now well characterized, especially those signals that are contained within transient N-terminal presequences (Schatz, 1987; Glick et al., 1992a). Recently there has been rapid progress in identifying components of the protein-translocation machinery (Baker & Schatz, 1991; Hannavy et al., 1993). Other studies have attempted to define the energy-dependent steps of import (Beasley et al., 1992) and the pathways of protein sorting to the different mitochondrial compartments (Glick et al., 1992a; Segui-Real et al., 1992).

We have been investigating the sorting of cytochrome b_2 and cytochrome c_1 to the intermembrane space compartment. These proteins are synthesized with unusually

long presequences of 80 and 61 residues, respectively (Sadler et al., 1984; Guiard, 1985). In both cases the N-terminal portion of the presequence resembles a mitochondrial matrix-targeting signal; the C-terminal portion, which contains a stretch of uncharged amino acids, is responsible for targeting to the intermembrane space (van Loon et al., 1986, 1987; Jensen et al., 1992; Koll et al., 1992; Beasley et al., 1993; Schwarz et al., 1993). Cleavage of the presequence occurs in two steps, the first being catalyzed by the general matrix-processing peptidase, the second by a different protease at the outer face of the inner membrane (Gasser et al., 1982; Ohashi et al., 1982; Reid et al., 1982; Teintze et al., 1982; Behrens et al., 1991; Schneider et al., 1991). Although it is generally agreed that these bipartite presequences contain information for intramitochondrial sorting, there is less agreement about how mitochondria decode this information (reviewed in Glick et al. [1992a]). The "conservative sorting" model states that cytochromes b_2 and c_1 are first imported across the inner membrane by the usual matrix-targeting pathway and then are reexported to the intermembrane space by a second, bacterial-type translocation system (Hartl et al., 1987; Hartl &

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Neupert, 1990; Koll et al., 1992). Our results favor the alternative "stop-transfer" model, in which the C-terminal portion of the presequence arrests translocation through the inner membrane, so that the mature part of the protein crosses only the outer membrane to reach the intermembrane space (van Loon & Schatz, 1987; Glick et al., 1992b; Wachter et al., 1992).

To date the most stringent test of these two models has been to determine whether sorting can take place in ATP-depleted mitochondria. Depletion of matrix ATP allows precursor proteins to cross the outer membrane but prevents complete translocation of precursors across the inner membrane (Hwang & Schatz, 1989; Hwang et al., 1991; Rassow & Pfanner, 1991). Thus, a stop-transfer mechanism might operate independently of matrix ATP, whereas a conservative sorting pathway should require matrix ATP for the initial import across the inner membrane (Glick et al., 1992b; Koll et al., 1992). We showed previously that cytochrome c_1 is imported and processed normally in ATP-depleted mitochondria, indicating that this protein never passes through the matrix (Wachter et al., 1992). However, ATP-depleted mitochondria are unable to import cytochrome b_2 (Hwang & Schatz, 1989). This was the only result that could not be explained readily by the stop-transfer model (see Discussion). In the present study we have investigated the role of matrix ATP in import of cytochrome b_2 .

Our analysis revealed that this ATP requirement does not reflect transport of cytochrome b_2 across the inner membrane, but instead is due to a folded structure in the mature part of the precursor protein. Mature cytochrome b_2 (reviewed in Chapman et al. [1991]) consists of a heme-binding domain (residues 1–100), a flavin domain (residues 101–488), and a C-terminal tail (residues 489–511). The heme-binding domain, which is located immediately downstream of the presequence, folds tightly even in the precursor form of the protein. Translocation of this domain across the outer membrane appears to be the only ATP-dependent step in the cytochrome b_2 import pathway.

Results

Import of cytochrome b_2 requires matrix ATP but not external ATP

Incubation of the cytochrome b_2 precursor (p) with mitochondria resulted in the formation of intermediate (i) and mature (m) forms of the protein (Fig. 1A). When the intact mitochondria (M) were then treated with proteinase K, a fraction of the processed molecules remained undigested, indicating that they had completely crossed the outer membrane. The imported forms of cytochrome b_2 faced the intermembrane space because they became accessible to protease when the outer membrane was rup-

tured by osmotic shock to generate mitoplasts (MP; Glick et al., 1992b).

ATP is necessary for import of cytochrome b_2 (Hartl et al., 1988). To determine the site of action of ATP, we used conditions that cause selective depletion of nucleotide levels either outside the inner membrane or in the matrix (Wachter et al., 1992). External ATP can be consumed by adding an enzymatic trap such as hexokinase plus glucose. This treatment inhibits import of certain precursors, including cytochrome c_1 (Wachter et al., 1992; Fig. 1A). Alternatively, inactivating the mitochondrial ATP synthase allows matrix ATP to be hydrolyzed by a potent endogenous ATPase activity (Fig. 1C). Depletion of matrix ATP does not block assembly of the adenine nucleotide translocator into the inner membrane (Wachter et al., 1992; Fig. 1B) but does arrest protein translocation across the inner membrane (Hwang et al., 1991; see below). In the case of cytochrome b_2 , depleting external ATP had only a weak inhibitory effect on import (Fig. 1A; Hwang & Schatz, 1989), whereas depleting matrix ATP blocked import completely (Fig. 1B).

A novel translocation intermediate of cytochrome b_2 accumulates in ATP-depleted mitochondria

After incubation with ATP-depleted mitochondria, cytochrome b_2 was associated with the membranes as the uncleaved precursor form (Fig. 2A, lane 1). The bound precursor was accessible to protease (lane 2), indicating that at least part of the polypeptide remained outside the outer membrane. This precursor form was a productive intermediate on the sorting pathway because restoration of ATP levels led to the generation of mature cytochrome b_2 (lanes 4, 6) that was localized to the intermembrane space (lanes 8, 9). We interpret these data as follows: in ATP-depleted mitochondria, the cytochrome b_2 presequence interacts with the import machinery but does not insert to its final position and therefore cannot be cleaved by the matrix-processing protease. However, at this stage the presequence is probably already associated with the inner membrane because completion of import takes place when matrix ATP is regenerated without changing any of the other incubation conditions (lane 4).

The ATP-induced import of the bound cytochrome b_2 precursor was blocked by the uncoupler carbonyl cyanide p -(trifluoromethoxy)phenylhydrazone (FCCP; Fig. 2A, lanes 5, 7), indicating that this chase reaction required an electrochemical potential ($\Delta\Psi$) across the inner membrane. In contrast, previously identified import intermediates that had interacted with the inner membrane were beyond the membrane potential-dependent step of translocation (Schleyer & Neupert, 1985; Hwang et al., 1991). For example, the matrix-targeted precursor Su9-dihydrofolate reductase (DHFR) accumulated in ATP-depleted mitochondria as a once-cleaved form spanning the inner membrane (Fig. 2A, lane 2; Manning-Krieg et al., 1991;

Fig. 1. Import of cytochrome *b*₂ requires matrix ATP but not external ATP. **A:** ATP levels were depleted outside the inner membrane. **B:** ATP levels were depleted in the matrix. In each case, control incubations included ATP on both sides of the inner membrane. A mixture of radiolabeled precursors, either (A) cytochrome *b*₂ plus cytochrome *c*₁ or (B) cytochrome *b*₂ plus the adenine nucleotide translocator, was incubated with mitochondria for 10 min at 15 °C. This time point was in the linear range for import of cytochrome *b*₂ (not shown). Aliquots of the mitochondria (M) were then treated with proteinase K (Prot. K), or converted to mitoplasts (MP) in the presence or absence of proteinase K. Finally, all samples were precipitated with trichloroacetic acid and subjected to SDS-PAGE. The upper part of each figure is a fluorograph of the dried gel. Portions of each sample were subjected to immunoblotting using antibodies against α -ketoglutarate dehydrogenase (KDH) and preexisting cytochrome *b*₂ (cyt. *b*₂). In B, 100% values for these marker proteins were defined as the amounts present in the non-protease-treated mitochondria. STD: 12.5% of the amount of precursor mixture initially added to each sample; p, i, m: precursor, intermediate, and mature forms of the two cytochromes; fragment: protease-resistant fragment of the assembled adenine nucleotide translocator (Wachter et al., 1992). **C:** Free matrix ATP is rapidly hydrolyzed after inhibition of the ATP synthase. Mitochondria (0.25 mg/mL) were incubated 3 min at 25 °C in standard import buffer containing 10 mM glycerol, 10 U/mL *E. coli* glycerokinase, 25 μ g/mL carboxyatractylide, and 2 mM NADH. This treatment depleted ATP outside the inner membrane but allowed synthesis of matrix ATP by oxidative phosphorylation. After cooling the mixture to 0 °C, oligomycin and efrapeptin were added to final concentrations of 5 and 2 μ g/mL, respectively. A sample was removed and precipitated with perchloric acid (see Materials and methods); an identical sample was adjusted to 0.2% Triton X-100 and precipitated with perchloric acid after 5 min on ice. The remaining mitochondria were placed at 30 °C. At the indicated time points duplicate samples were taken for immediate acid precipitation or detergent treatment followed by acid precipitation. The overall ATP concentration in the samples containing intact mitochondria (●), and the concentration of protein-bound ATP as measured using detergent-treated mitochondria (○) are shown (see Materials and methods). The concentration of free matrix ATP for the zero time point was approx. 0.85 mM. More than 90% of this ATP was hydrolyzed within the first minute at 30 °C. Although the presence of bound ATP makes it difficult to determine accurately the free ATP concentration for later time points, we estimate that this value was less than 10 μ M after 5 min at 30 °C.

Wachter et al., 1992), and conversion of this species to the fully imported mature form required ATP but not $\Delta\Psi$ (lanes 3–5). Thus, the cytochrome *b*₂ precursor that was partially inserted into ATP-depleted mitochondria represents a new type of translocation intermediate.

When trypsin was added to mitochondria that had accumulated this translocation intermediate of cytochrome *b*₂, a 10.5-kDa “core” fragment was generated from the bound precursor (Fig. 2B). As described below, the presence of this protease-resistant fragment indicates that the entire mature portion of the cytochrome *b*₂ precursor remained outside the outer membrane of ATP-depleted mitochondria.

Urea denaturation allows cytochrome *b*₂ to follow the correct sorting pathway even in ATP-depleted mitochondria

If the cytochrome *b*₂ precursor was denatured in 8 M urea before being added to mitochondria, it was rapidly imported (Fig. 3A). Import of the denatured precursor was unaffected by depletion of external ATP (not shown). When ATP was depleted either in the matrix (not shown) or on both sides of the inner membrane (Fig. 3A), the import efficiency was still relatively high, about 40% of that in fully energized mitochondria. ATP depletion had no effect on the kinetics of import. Thus, urea denaturation

cytochrome b_2 precursor. ATP-depleted mitochondria were incubated with cytochrome b_2 precursor as in part A. The mitochondria were then sedimented and resuspended at a concentration of 1 mg/mL in import buffer lacking bovine serum albumin (BSA), and the mixture was divided in half. One portion received trypsin (0.5 mg/mL) and both portions were incubated for 12 min at 23 °C. Soybean trypsin inhibitor (SBTI; 1 mg/mL) was then added to both portions, and the mitochondria were centrifuged for 3 min at 12,000 $\times$ g . The membrane pellet was resuspended in the same buffer containing SBTI but no BSA. Pellet (Pel) and supernatant (Sup) fractions were subjected to trichloroacetic acid precipitation followed by SDS-PAGE and fluorography. MW STD: the numbers indicate the molecular masses in kDa of reference proteins. The band immediately below the cytochrome b_2 precursor is the pseudomature form, which is often produced during in vitro translation by initiation at position +6 of the mature protein (Guiard, 1985). After trypsin treatment, the radioactivity recovered in the 10.5-kDa core fragment was 24% of that initially present in the bound precursor, indicating that the heme-binding domain was folded in essentially all of these precursor molecules (see Materials and methods).

enabled some of the cytochrome b_2 molecules to bypass the matrix ATP requirement for import.

In ATP-depleted mitochondria, the imported forms of cytochrome b_2 were exposed to the intermembrane space (Fig. 3B). It is unlikely that these molecules had followed an import-reexport pathway because ATP depletion prevented translocation of a matrix-targeted precursor across the inner membrane in the same experiment (Fig. 3B, Su9-DHFR). We therefore asked whether cytochrome b_2 was using its normal sorting pathway under these conditions. Useful criteria for correct targeting to the intermembrane space are proper folding after import and cleavage to the

Fig. 2. Depleting matrix ATP causes accumulation of a novel translocation intermediate of cytochrome b_2 . **A:** As in Figure 1B, mitochondria were selectively depleted of matrix ATP while maintaining an electrochemical potential ($\Delta\Psi$) across the inner membrane. The mixture was divided in half: one portion was incubated with the cytochrome b_2 precursor for 8 min at 25 °C (binding reaction), and the other portion was incubated with the Su9-DHFR precursor under identical conditions. Each portion was then processed as follows. The mitochondria were sedimented, resuspended in standard import buffer containing 10 mM 2-deoxyglucose, and divided into nine equal samples. Two samples were left on ice (lanes 1, 2). The remaining samples were subjected to a chase reaction for 4 min at 30 °C under various conditions of energization of the mitochondria. The membrane potential was either maintained by adding NADH to 1 mM or else dissipated by adding FCCP to 25 μ M. Chase reactions included either 10 U/mL hexokinase (no ATP), 10 mM α -ketoglutarate plus 10 U/mL hexokinase (ATP in), or 10 mM α -ketoglutarate plus 0.5 mM ATP (ATP in + out). Mitochondria (M) were then treated with proteinase K (Prot. K; lanes 2–7), or converted to mitoplasts (MP) in the presence of proteinase K (lanes 8, 9). All samples were precipitated with trichloroacetic acid and subjected to SDS-PAGE and fluorography. A portion of each sample was used for immunoblotting of endogenous marker proteins as in Figure 1. The numbers indicate the percentages of the marker proteins recovered, where 100% is defined as the amounts for the non-protease-treated sample. The values listed correspond to the experiment involving the cytochrome b_2 precursor; recovery of marker proteins in the parallel experiment with Su9-DHFR was essentially identical (not shown). STD: precursor standards (5% for cytochrome b_2 , 10% for Su9-DHFR); p, i, m: precursor, intermediate, and mature forms of cytochrome b_2 and Su9-DHFR. **B:** A 10.5-kDa “core” fragment was generated by trypsin digestion of the surface-bound

mature form by the inner membrane protease (Wachter et al., 1992). With cytochrome b_2 , these two criteria are related because efficient cleavage requires partial folding of the imported polypeptide. This effect is illustrated in Figure 4; maturation of cytochrome b_2 was accelerated by hemin, which presumably interacts with the heme-binding domain of the protein after this domain has assumed its native conformation. Hemin stimulated cleavage both in fully energized and in ATP-depleted mitochondria (Fig. 4). Folding of the heme-binding domain was also assessed by a more direct method. When the endogenous (preexisting) cytochrome b_2 was released from the mito-

A. Mitochondria + protease

B. Mitoplasts + protease

Fig. 3. Urea-denatured cytochrome *b*₂ precursor is imported into ATP-depleted mitochondria. A mixture of the cytochrome *b*₂ and Su9-DHFR precursors was denatured in 8 M urea and diluted into import buffer containing either fully energized or ATP-depleted mitochondria. After incubation at 18 °C for the indicated times, aliquots were removed, adjusted to 25 μ M FCCP, and placed on ice. In each case, a control aliquot received 25 μ M FCCP before addition of the precursors. One sample from each aliquot was treated with proteinase K (A); a second sample was converted to mitoplasts in the presence of proteinase K (B). All samples were precipitated with trichloroacetic acid and subjected to SDS-PAGE and fluorography. A portion of each sample was used for immunoblotting of endogenous marker proteins as in Figure 1. The 100% levels for marker proteins were defined as the average of the amounts in samples of protease-treated intact mitochondria. STD: 20% precursor standards; p, i, m: as in Figure 2A.

chondria and then digested with trypsin, the heme-binding domain was recovered as a protease-resistant 10.5-kDa core fragment (Fig. 5; Guiard & Lederer, 1976). The same fragment was generated by trypsin treatment of radiolabeled cytochrome *b*₂ that had been imported into either fully energized or ATP-depleted mitochondria (Fig. 5). Approximately 20% of the radioactivity present in the imported mature form could be recovered in the 10.5-kDa species. Because this fragment should contain two of the

Fig. 4. Hemin stimulates maturation of cytochrome *b*₂ that has been imported into fully energized or ATP-depleted mitochondria. Parallel experiments were performed with the cytochrome *b*₂ and Su9-DHFR precursors (see Fig. 2A). In each case, urea-denatured precursor was diluted into import buffer containing either fully energized or ATP-depleted mitochondria. Where indicated, the import buffer contained 2 μ M hemin. After incubation for 2 or 10 min at 18 °C, aliquots were removed, adjusted to 25 μ M FCCP, and transferred to ice. The mitochondria were then treated with proteinase K or converted to mitoplasts in the presence of proteinase K. All samples were precipitated with trichloroacetic acid and subjected to SDS-PAGE and fluorography. As in Figure 1, a portion of each sample was used for immunoblotting of endogenous marker proteins, which confirmed that the outer membrane had been selectively ruptured during mitoplast formation (not shown). STD: 10% precursor standards; p, i, m: as in Figure 2A. Hemin also stimulated maturation when the nondenatured cytochrome *b*₂ precursor was imported into fully energized mitochondria (not shown).

nine methionine residues in the mature protein (see Materials and methods), the heme-binding domain was folded in essentially all of the imported molecules. Taken together, these data suggest that depletion of matrix ATP did not alter the topology or the conformation of imported cytochrome *b*₂.

When endogenous cytochrome *b*₂ was digested with trypsin, several distinct fragments were generated in addition to the 10.5-kDa core (Fig. 5). This proteolysis pattern is characteristic of the fully folded tetrameric protein (Gervais & Tegoni, 1980). In contrast, trypsin treatment of newly imported cytochrome *b*₂ yielded only the core fragment. Upon further incubation, however, some of the imported molecules acquired digestion characteristics of the native tetramer (Fig. 5). This *in vitro* assembly reaction occurred with a half-time of about 30 min at 25 °C (not shown). Only 10–15% of the imported cytochrome *b*₂ became assembled, probably because oligomerization was limited by the small number of radiolabeled molecules that would have been present in each mitochondrion. The efficiency of assembly was identical after import into fully energized or ATP-depleted mitochondria (Fig. 5), further indicating that cytochrome *b*₂ follows its normal sorting pathway when the matrix is depleted of ATP.

Matrix ATP drives translocation of the folded heme-binding domain

An attractive hypothesis is that matrix ATP is needed to "pull" the folded heme-binding domain of cytochrome

trichloroacetic acid and subjected to SDS-PAGE and fluorography (left). A portion of each sample was used for immunoblotting with a polyclonal anti-cytochrome b_2 antiserum (right). Precursor and mature forms of radiolabeled cytochrome b_2 are indicated by p and m, respectively; shorter exposures of the fluorographs revealed that essentially all of the imported cytochrome b_2 was present as the mature form after the initial 15 °C incubation (not shown). Core: 10.5-kDa tryptic fragment from the heme-binding domain. Arrows indicate additional tryptic fragments derived from assembled cytochrome b_2 . The relative intensities of these bands depend upon their methionine content in the case of the radiolabeled protein and upon the presence of immunoreactive epitopes in the case of endogenous cytochrome b_2 . The band marked with an asterisk (*) probably corresponds to the fragment previously referred to as β' (Gervais & Tegoni, 1980). By quantifying the amount of β' obtained after treatment with 5 $\mu\text{g/ml}$ trypsin, we estimated that the assembly efficiency was 14% after import into fully energized mitochondria and 12% after import into ATP-depleted mitochondria (see Materials and methods).

b_2 across the outer membrane. Figure 6A shows that the isolated heme-binding domain is extremely stable, with a T_m of greater than 80 °C as measured by differential scanning calorimetry. The heme-binding domain can fold independently of the rest of the molecule (Brunt et al., 1992), suggesting that it might be folded even in the precursor form of the protein. Indeed, trypsin digestion of the radiolabeled cytochrome b_2 precursor gave a 10.5-kDa core fragment (Fig. 6B). As shown above, the same tryptic fragment was obtained after the precursor had been incubated with ATP-depleted mitochondria (Fig. 2B), confirming that the heme-binding domain remained folded and did not cross the outer membrane under these conditions.

Fig. 5. Cytochrome b_2 folds and assembles correctly after import into ATP-depleted mitochondria. Urea-denatured cytochrome b_2 precursor was diluted 40-fold into import buffer containing 2 μM hemin and 50 $\mu\text{g/ml}$ of (A) fully energized or (B) ATP-depleted mitochondria. After 10 min at 15 °C, FCCP was added to 25 μM and the mixture was transferred to ice. To facilitate subsequent manipulations, the mitochondrial protein concentration was raised to 150 $\mu\text{g/ml}$ by addition of FCCP-treated mitochondria. The mitochondria were then sedimented and resuspended at a concentration of 1 mg/ml in import buffer lacking BSA and supplemented with 2 mM NADH, 2 μM hemin, 10 μM FMN, 25 μM FCCP, 1 mM ATP, 10 mM creatine phosphate, and 0.1 mg/ml rabbit muscle creatine kinase. The mixture was divided in half; one portion was left on ice and the other portion was subjected to a 1-h chase reaction at 30 °C. A sample (M) corresponding to 25% of each portion was treated with 100 $\mu\text{g/ml}$ trypsin for 20 min on ice, followed by addition of SBTI to 1 mg/ml. The remaining mitochondria were converted to mitoplasts by osmotic shock and centrifuged for 5 min at 12,000 $\times g$. The supernatants containing soluble intermembrane space proteins (IMS) were divided into three equal samples and treated with 0, 5, or 100 $\mu\text{g/ml}$ trypsin for 20 min on ice, followed by addition of SBTI to 1 mg/ml. All samples were then precipitated with

This hypothesis explains why the urea-treated cytochrome b_2 precursor could be imported into ATP-depleted mitochondria: the heme-binding domain was presumably denatured. However, matrix ATP still stimulated import of the denatured precursor. It may be that the heme-binding domain refolded in some of these precursor molecules before they had a chance to cross the outer membrane. Alternatively, matrix ATP might have a second function in import of cytochrome b_2 apart from translocation of the heme-binding domain. To distinguish between these possibilities we created the cytochrome b_2 - $\Delta 14$ protein, in which the heme-binding domain has been permanently destabilized by a 14-amino acid deletion (Fig. 7; Kinemage 1). As expected, trypsin treatment of

Fig. 6. The heme-binding domain of cytochrome *b*₂ is tightly folded, even in the precursor form of the protein. **A:** The isolated cytochrome *b*₂ heme-binding domain was subjected to differential scanning calorimetry as described in Materials and methods. The heme-binding domain began to unfold at about 80 °C; further unfolding was accompanied by irreversible aggregation and precipitation of the protein. **B:** Folding of the heme-binding domain can be monitored by trypsin treatment. Radiolabeled precursors of cytochrome *b*₂, cytochrome *b*₂-Δ14, pb₂(1-167)-DHFR, pb₂(1-185)-DHFR, and pb₂(1-180) were adjusted to 20 μM methotrexate, and aliquots were incubated with 0, 50, or 500 μg/mL trypsin for 20 min on ice. Proteins were then precipitated with trichloroacetic acid and subjected to SDS-PAGE and fluorography. The asterisks (*) mark the precursor forms of the proteins. MW STD: the numbers indicate the molecular masses in kDa of reference proteins. Core: 10.5-kDa tryptic fragment of the heme-binding domain. The measured recovery of radioactivity in the core fragments after digestion with 500 μg/mL trypsin was 15% for pre-cytochrome *b*₂, 16% for pb₂(1-185)-DHFR, and 55% for pb₂(1-180); thus, the heme-binding domain was folded in essentially all of these precursor molecules (see Materials and methods). With cytochrome *b*₂-Δ14 and pb₂(1-167)-DHFR, no low-molecular mass fragments were detected even after prolonged exposure of the fluorogram (not shown). The DHFR domain was protease resistant and therefore folded in both pb₂(1-167)-DHFR and pb₂(1-185)-DHFR (Endo et al., 1989).

Fig. 7. Structure of the heme-binding domain of cytochrome *b*₂ as determined by Xia and Mathews (1990). The diagram shows amino acids 1-100 of mature cytochrome *b*₂ (or residues 81-180 of the precursor form). Arrows represent β -strands and cylinders represent helices; the heme molecule is shown in outline. The shaded region corresponds to the 14-amino acid deletion in cytochrome *b*₂-Δ14. Small arrow indicates the position at which fusion to DHFR occurs in pb₂(1-167)-DHFR.

this mutant precursor did not yield a protease-resistant core fragment (Fig. 6B). Cytochrome *b*₂-Δ14 was imported to the intermembrane space and cleaved by the matrix-processing protease (Fig. 8A,B), but maturation was slow and was not stimulated by hemin (Fig. 8C), further indicating that the heme-binding domain is disrupted in this protein. Import and processing of cytochrome *b*₂-Δ14 were unaffected by depletion of matrix ATP (Fig. 8A). These results suggest that the folded heme-binding domain of cytochrome *b*₂ is entirely responsible for the ATP dependence of import.

Import of artificial precursors containing an intact cytochrome b₂ heme-binding domain requires ATP only in the matrix

Previous studies examined the role of ATP in import of fusion proteins that contain truncated forms of the cytochrome *b*₂ precursor joined to DHFR (Pfanner et al., 1990; Glick et al., 1992b). Depletion of ATP had no effect on import of the precursor pb₂(1-167)-DHFR, which includes only the first 87 residues of mature cytochrome *b*₂, but strongly inhibited import of longer fusion

Fig. 8. Disruption of the heme-binding domain of cytochrome b_2 allows import into ATP-depleted mitochondria. STD: 20% precursor standard; p, i, m: precursor, intermediate, and mature forms of the radiolabeled proteins. **A, B:** A mixture of the cytochrome b_2 - $\Delta 14$ and Su9-DHFR precursors was added to fully energized or ATP-depleted mitochondria. After incubation at 25 °C for the indicated times, samples were removed and adjusted to 25 μ M FCCP. Proteinase K treatment of mitochondria or mitoplasts and immunoblotting for endogenous marker proteins were performed as in Figure 3. A parallel control experiment confirmed that import of wild-type cytochrome b_2 was blocked in the ATP-depleted mitochondria (not shown). Depletion of external ATP had no significant effect on import of cytochrome b_2 - $\Delta 14$ (not shown). **C:** A mixture of the cytochrome b_2 - $\Delta 14$ and $pb_2(1-167)$ -DHFR precursors was added to mitochondria in standard import buffer plus 2 mM ATP, in the absence or presence of 2.5 μ M hemin. After 5, 10, or 20 min at 25 °C, samples were adjusted to 25 μ M FCCP, treated with proteinase K, precipitated with trichloroacetic acid, and subjected to SDS-PAGE and fluorography. STD: 15% precursor standards.

proteins. We reexamined these findings in light of the possible influence of the heme-binding domain. $pb_2(1-167)$ -DHFR is targeted to the intermembrane space and processed in two steps like authentic cytochrome b_2 (Rasow et al., 1989; Glick et al., 1992b; Koll et al., 1992). However, $pb_2(1-167)$ -DHFR has an incomplete heme-binding domain (Fig. 7; Kinemage 1). This truncated domain is still able to bind heme, as maturation of $pb_2(1-167)$ -DHFR was stimulated by hemin (Fig. 8C); depletion of matrix ATP had only a mild inhibitory effect on import of $pb_2(1-167)$ -DHFR (Fig. 9A; Glick et al., 1992b), suggesting that the heme-binding domain is destabilized in this fusion protein. As expected, trypsin digestion of the $pb_2(1-167)$ -DHFR precursor did not yield a protease-resistant core fragment (Fig. 6B). An intact heme-binding domain is present in the slightly longer fusion construct $pb_2(1-185)$ -DHFR (Fig. 6B), and import of this protein required matrix ATP (Fig. 9A). A similar ATP dependence was found for import of $pb_2(1-180)$ (Fig. 9B), which consists of the presequence plus the complete heme-binding domain of cytochrome b_2 (Brunt et al., 1992; Fig. 6B).

Depletion of external ATP did not prevent import of the fusion proteins $pb_2(1-167)$ -DHFR and $pb_2(1-185)$ -

DHFR (Fig. 9C) or of $pb_2(1-180)$ (not shown). As a control, import of the adenine nucleotide translocator was strongly inhibited under these conditions (Fig. 9C; Wachter et al., 1992). For $pb_2(1-185)$ -DHFR, the amount of mature form was actually increased by depletion of external ATP; this effect is apparently due to an ATP-dependent proteolytic activity in the intermembrane space (Glick et al., 1992b, and unpubl. data). Thus, import of several precursors that contain the intact cytochrome b_2 heme-binding domain requires ATP in the matrix but not outside the inner membrane.

Late stages in the import pathway of $pb_2(1-167)$ -DHFR are independent of $\Delta\Psi$

Both cytochrome b_2 and $pb_2(1-167)$ -DHFR require an electrochemical potential ($\Delta\Psi$) across the inner membrane for import (Hartl et al., 1987; Rasow et al., 1989). With cytochrome b_2 , $\Delta\Psi$ seems to be needed only for the initial insertion of the presequence into the inner membrane (Hartl et al., 1987; Pfanner et al., 1987a). However, it was suggested (Koll et al., 1992) that $\Delta\Psi$ might also act later in the sorting pathway of $pb_2(1-167)$ -DHFR, during a putative reexport step; this reaction would be analogous

Fig. 9. Depletion of matrix ATP blocks import of precursors that contain the folded heme-binding domain of cytochrome *b*₂. STD: 10% precursor standards; p, i, m: precursor, intermediate, and mature forms of the radiolabeled proteins. Import times were within the linear range for the precursors containing the cytochrome *b*₂ presequence (not shown). All reaction mixtures contained 2.5 μ M hemin. **A:** Precursors of pb₂(1-167)-DHFR, pb₂(1-185)-DHFR, and Su9-DHFR were incubated with fully energized mitochondria (i + o) or with mitochondria selectively depleted of matrix ATP (out) for 15 min at 25 °C. After import, mitochondria (M) were treated with 500 μ g/mL proteinase K for 30 min on ice or converted to mitoplasts (MP) in the presence of 100 μ g/mL proteinase K. All samples were then precipitated with trichloroacetic acid and subjected to SDS-PAGE and fluorography. A portion of each sample was used for immunoblotting of endogenous marker proteins as in Figure 1. The 100% levels for marker proteins were defined as the amounts present in protease-treated intact mitochondria from each reaction. **B:** The pb₂(1-180) precursor was incubated with fully energized or ATP-depleted mitochondria for 2 min at 15 °C. Further processing and analysis were performed as in Figure 1B. **C:** Precursors of pb₂(1-167)-DHFR, pb₂(1-185)-DHFR, and the adenine nucleotide translocator were incubated for 15 min at 25 °C with fully energized mitochondria (i + o) or with mitochondria selectively depleted of external ATP (in). Further processing and analysis were performed as in part A. Fragment: see Figure 1B.

to bacterial protein export, which can be driven by a proton-motive force in the absence of ATP (Driessen, 1992). Thus, according to the conservative sorting model, $\Delta\Psi$ might substitute for matrix ATP as the energy source for a coupled import-reexport mechanism. To test this possibility, we first incubated pb₂(1-167)-DHFR with mitochondria at 12 °C in the presence of $\Delta\Psi$. Under these conditions the presequence inserted fully into the inner membrane and was cleaved by the matrix-processing peptidase, whereas the DHFR domain remained outside the outer membrane and accessible to proteinase K (Fig. 10). A subsequent chase at 25 °C led to import of pb₂(1-167)-DHFR to the intermembrane space (Fig. 10). Dissipating $\Delta\Psi$ had no effect on the kinetics of the chase reaction (Fig. 10A), even when the matrix was depleted of ATP (Fig. 10B). To confirm that $\Delta\Psi$ and matrix ATP had been eliminated, we added the Su9-DHFR precursor at the beginning of the chase incubations; as expected, abolishing $\Delta\Psi$ completely blocked import of Su9-DHFR, and depleting matrix ATP arrested passage of this precursor across the inner membrane (Fig. 10). The combined results indicate that $\Delta\Psi$ is not involved in translocation of

the DHFR portion of pb₂(1-167)-DHFR to the intermembrane space.

Discussion

Cytochrome *b*₂ and its fusion protein derivatives have been used to study many aspects of mitochondrial protein import, including the nature of translocation contact sites (Pfanner et al., 1987a; Rassow et al., 1989, 1990), the structure of intermembrane space targeting signals (Koll et al., 1992; Beasley et al., 1993; Schwarz et al., 1993), and the role of ATP and chaperone proteins in translocation (Hartl et al., 1988; Cheng et al., 1989; Hwang & Schatz, 1989; Kang et al., 1990; Pfanner et al., 1990; Glick et al., 1992b; Koll et al., 1992; Hallberg et al., 1993; Schwarz et al., 1993). The interpretation of much of this work depends upon the mechanism by which cytochrome *b*₂ is sorted to the intermembrane space. Hartl et al. (1987) concluded that cytochrome *b*₂ is initially imported completely across the inner membrane, but our investigations have revealed no evidence for a sorting intermediate in the matrix (Glick et al. [1992b] and this

A. Matrix ATP

B. No Matrix ATP

Fig. 10. Dissipating $\Delta \psi$ does not inhibit translocation of the DHFR domain of $pb_2(1-167)$ -DHFR across the outer membrane, even in ATP-depleted mitochondria. The $pb_2(1-167)$ -DHFR precursor was incubated with mitochondria in standard import buffer plus 2 mM NADH for 10 min at 12 °C. The mitochondria were then sedimented, resuspended in standard import buffer containing 2 mM NADH and 2.5 μ M hemin, and divided into two portions. Both portions received 10 U/mL apyrase to consume external ATP. In one portion (A), matrix ATP was generated by adding 20 μ g/mL carboxyatractylsine and 5 mM α -ketoglutarate; in the other portion (B), matrix ATP was depleted by adding 5 μ g/mL oligomycin and 2 μ g/mL efrapeptin. Both portions were incubated a further 10 min at 12 °C and then placed on ice. Two samples of the mitochondria (M) were removed; one was left untreated and the other was treated with proteinase K. The remainder of each portion was divided into two aliquots. One aliquot received 25 μ M FCCP and 1 μ M valinomycin to dissipate the electrochemical potential ($\Delta \psi$) and proton-motive force across the inner membrane. Su9-DHFR precursor (pretreated with 50 U/mL apyrase for 5 min at 30 °C) was then added to both aliquots, and the tubes were immediately transferred to 25 °C. Samples were removed after 8, 16, or 24 min at 25 °C. These samples were either treated with proteinase K or converted to mitoplasts (MP) in the presence of proteinase K. All samples were then precipitated with trichloroacetic acid and subjected to SDS-PAGE and fluorography. A portion of each sample was used for immunoblotting of endogenous marker proteins as in Figure 1. The 100% levels for the marker proteins were defined as the average of the amounts present in the mitochondrial samples. STD: 20% precursor standards; p, i, m: see Figure 9. Dissipating $\Delta \psi$ weakly inhibited the second cleavage of the $pb_2(1-167)$ -DHFR presequence; a longer exposure of the fluorogram confirmed that the intermediate-sized form that remained after the 25 °C chase was exposed to the intermembrane space (not shown). Following the 12 °C preincubation and subsequent protease treatment, the intact DHFR moiety was released from the mitochondria (not shown).

study) even when experiments were performed under the same reaction conditions as in the original report (B. Glick, unpubl.). We showed that some of the earlier analysis methods failed to distinguish between intermediate forms in the matrix and those bound to the outer face of the inner membrane (Glick et al., 1992b). Another approach for examining the sorting of cytochrome b_2 has been to determine whether this precursor ever associates with the matrix-localized hsp60 chaperonin. Although preliminary evidence for such an interaction was obtained by inactivating hsp60 in living yeast cells (Cheng et al., 1989), subsequent *in vivo* studies indicated that hsp60 function is not required for cytochrome b_2 to reach the intermembrane space (Glick et al., 1992b; Hallberg et al., 1993). In experiments with isolated mitochondria, imported fusion proteins containing the cytochrome b_2 presequence were reportedly associated with hsp60 (Koll et al., 1992); however, we find that the techniques used to monitor this association can give inaccurate results (S. Rospert & B. Glick, in prep.). In summary, there is no indication that cytochrome b_2 passes through the matrix.

The results described above do not exclude the possibility that cytochrome b_2 is imported across the inner membrane and then rapidly reexported by an hsp60-independent mechanism. Such a sorting pathway would still require matrix ATP. The experimental data on this point have been confusing because ATP-depleted mitochondria can import the fusion protein $pb_2(1-167)$ -DHFR (Pfanner et al., 1990; Glick et al., 1992b; Schwarz et al., 1993), but not authentic cytochrome b_2 (Hwang & Schatz [1989] and this study). The present work suggests an explanation for this discrepancy: matrix ATP is needed for import of authentic cytochrome b_2 because the presequence must be "pulled" far enough into the mitochondria for the stop-transfer signal to be recognized. In the absence of such a pulling force, the folded heme-binding domain remains outside the outer membrane and prevents the presequence from inserting to its final position (Fig. 11). The evidence for this model is as follows: (1) The heme-binding domain is very stable, as judged by its resistance to proteolysis and to thermal denaturation. Protease digestion studies confirm that this domain is folded even in the precursor form of cytochrome b_2 . (2) When the cytochrome b_2 precursor is incubated with ATP-depleted mitochondria, the heme-binding domain remains folded and does not cross the outer membrane. (3) Urea denaturation of the cytochrome b_2 precursor allows for import, processing, and assembly in ATP-depleted mitochondria. (4) When the heme-binding domain is disrupted by a 14-amino acid deletion, import is no longer affected by depletion of matrix ATP. (5) $pb_2(1-167)$ -DHFR has a truncated and destabilized heme-binding domain and can be imported into ATP-depleted mitochondria. $pb_2(1-185)$ -DHFR and $pb_2(1-180)$ contain an intact heme-binding domain, and import of these precursors requires matrix ATP.

Fig. 11. Proposed import pathway of cytochrome b_2 . OM: outer membrane; IM: inner membrane. The precursor, shown at the upper left, consists of a two-part presequence (the wavy line with + signs represents the amino-terminal part of the presequence, and the black rectangle represents the intermembrane space targeting signal), a heme-binding domain (circle), and a flavin domain (long wavy line). *Step 1:* The presequence inserts into the two membranes, but complete insertion is prevented by the folded heme-binding domain. This step is presumed to require an electrochemical potential ($\Delta\Psi$) across the inner membrane. *Step 2:* Matrix ATP is used to "pull" the precursor a short distance into the organelle, allowing the heme-binding domain to cross the outer membrane as the stop-transfer signal engages the sorting machinery in the inner membrane. This step also requires $\Delta\Psi$. Mitochondrial hsp70 (mhsp70) may act as the ATP-dependent import motor. *Step 3:* The amino-terminal part of the presequence is removed by the matrix-processing peptidase, and the mature protein completes its translocation across the outer membrane. The flavin domain (rounded diamond) may fold either at this stage, as shown, or else during a later assembly reaction. *Step 4:* The second part of the presequence is removed by inner membrane protease 1 (Behrens et al., 1991; Schneider et al., 1991). Cleavage is stimulated by the association of heme (modified square) with the heme-binding domain. *Step 5:* Mature cytochrome b_2 assembles into a tetramer.

Thus, translocation of the heme-binding domain across the outer membrane is the ATP-dependent step in import of cytochrome b_2 . The sorting reaction, which occurs during or after translocation of the heme-binding domain, is unaffected by depletion of matrix ATP. Additional experiments indicated that the electrochemical potential ($\Delta\Psi$) across the inner membrane acts only during the early stages of the cytochrome b_2 import pathway (Fig. 11). These results are incompatible with conservative sorting as originally proposed (Hartl & Neupert, 1990; Koll et al., 1992). In order to explain the experimental findings with a modified conservative sorting model, one would have to postulate that cytochrome b_2 departs from the normal matrix import pathway and immediately engages a re-export system that utilizes either protein-bound ATP, low levels of free ATP, or no ATP at all (Schwarz et al., 1993). This explanation seems unlikely because our depletion protocol severely reduces the concentration of free matrix ATP, and a bacterial-type export reaction would be

expected to involve multiple rounds of ATP hydrolysis by a single ATPase molecule (Driessen, 1992; Arkowitz et al., 1993). We prefer the more straightforward interpretation: because depletion of matrix ATP blocks protein import across the inner membrane but does not affect sorting of cytochrome b_2 , this precursor follows a stop-transfer pathway (Fig. 11).

Although matrix ATP clearly drives translocation of the heme-binding domain across the outer membrane, it is not obvious how it does so. The presence of ATP in the matrix appears to generate a pulling force that acts on the presequence. A logical candidate for an ATP-dependent "import motor" is mitochondrial hsp70 (mhsp70; Kang et al., 1990; Scherer et al., 1990; Manning-Krieg et al., 1991). It is conceivable that the heme-binding domain remains completely folded during translocation, as the mitochondrial import machinery can accommodate structures other than extended polypeptide chains (Vestweber & Schatz, 1988b). However, tightly folded proteins are

usually unable to cross the outer membrane (Eilers & Schatz, 1986; Chen & Douglas, 1987; Vestweber & Schatz, 1988a), suggesting that the heme-binding domain unfolds at least partially. If so, then matrix ATP might function in the following manner during import of cytochrome b_2 . At an early stage of import the precursor spans the two mitochondrial membranes with the heme-binding domain still folded at the surface of the outer membrane (Fig. 11). Like all protein structures, the heme-binding domain will undergo spontaneous partial unfolding events, which should allow the presequence to penetrate further into the matrix. Normally such oscillations are reversible, but matrix ATP would enable mhs70 to "capture" the presequence after an inward movement, thereby making unfolding irreversible and leading to vectorial translocation (Neupert et al., 1990; Simon et al., 1992; Arkowitz et al., 1993).

It has been suggested that ATP-dependent cytosolic chaperones facilitate translocation by preventing the tight folding of precursor proteins (Deshaies et al., 1988a; Rothman, 1989; Glick & Schatz, 1991). It is therefore striking that matrix ATP, not external ATP, is necessary for import of the heme-binding domain. The "antifolding" hypothesis also does not explain why precursors containing a DHFR moiety can fold after synthesis in a reticulocyte lysate and can then be imported in the absence of external ATP (Pfanner et al., 1990). The most dramatic example is the fusion protein pb₂(1-185)-DHFR: both the heme-binding domain and the DHFR domain fold in the reticulocyte lysate, and import is independent of external ATP. Thus, at least in some cases, mitochondria are capable of dealing with folded precursors without assistance from cytosolic ATPases. These observations support our previous suggestion that matrix ATP plays a more fundamental role than cytosolic ATP in mitochondrial protein import (Hwang & Schatz, 1989). On the other hand, ATP-dependent cytosolic chaperones presumably do help to prevent aggregation of some incompletely folded mitochondrial precursors. For example, external ATP strongly stimulates import of cytochrome c_1 and the adenine nucleotide translocator (Wachter et al., 1992), both of which probably fold only in the context of their association with the inner membrane. The stimulation by external ATP is much weaker for precursors of the soluble proteins cytochrome b_2 (Hwang & Schatz [1989] and this study) and hsp60 (C. Wachter, unpubl.). Of course, these experiments involve a heterologous *in vitro* system, and the effect of cytosolic chaperones might be more pronounced in the living cell.

Consideration of our results together with earlier published findings leads to the following general model for the role of cytosolic chaperones in eukaryotic protein translocation. Most precursors that enter the secretory pathway interact with the signal recognition particle (SRP) and then undergo cotranslational translocation into the endoplasmic reticulum (Nunnari & Walter, 1992). If

SRP fails to bind, chaperones of the hsp70 class associate with the nascent precursor as it emerges from the ribosome (Beckmann et al., 1990; Nelson et al., 1992). These hsp70 proteins initially function to prevent aggregation of newly synthesized polypeptide chains (Ellis & van der Vies, 1991). Additional chaperones (Atencio & Yaffe, 1992; Caplan et al., 1992), including those that specifically recognize organellar targeting sequences (Ono & Tuboi, 1990; Murakami et al., 1992; Hachiya et al., 1993), may bind during or after translation. Some precursors will remain unfolded and aggregation prone as long as they are in the cytosol and thus will be associated with chaperones until translocation occurs (Chirico et al., 1988; Deshaies et al., 1988b; Murakami et al., 1988; Zimmermann et al., 1988; Waegemann et al., 1990). Other precursors may be released from the chaperones in a partially folded form that is both resistant to aggregation and competent for translocation (Pilon et al., 1992; Lill et al., 1992; Miller & Cumsky, 1993). Still other precursors will be capable of partial or complete folding in the cytosol, but folding will be slow because association with the chaperones is relatively long-lived. This preservation of the unfolded state is probably essential for translocation of certain proteins (Mattingly et al., 1993). Finally, some precursors will dissociate from the chaperones and fold quite rapidly, but will unfold again during subsequent translocation (Skerjanc et al., 1990). Mitochondrial precursors that contain either a DHFR moiety or the cytochrome b_2 heme-binding domain probably belong in this last category (Eilers & Schatz, 1986; Pfanner et al., 1990; Wienhues et al., 1991; this study).

We now have a detailed and reasonably coherent picture of the energetics of protein import into mitochondria. Future work will be directed at identifying the ATPases that mediate import, and at understanding how the energy of ATP hydrolysis drives the translocation of polypeptides across biological membranes.

Materials and methods

General methods

Most procedures have been described elsewhere (Glick, 1991; Glick et al., 1992b; Wachter et al., 1992), including preparation of yeast mitochondria and measurement of mitochondrial protein concentration, osmotic shock treatment to generate mitoplasts (mitochondria whose outer membrane has been selectively ruptured but whose inner membrane remains intact), proteinase K treatment (50–100 μ g/mL protease for 20–30 min on ice), use of protease-sensitive markers for the matrix (α -ketoglutarate dehydrogenase) and intermembrane space (endogenous cytochrome b_2), precipitation with trichloroacetic acid, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, fluorography, laser densitometry, and immunoblotting with rabbit antisera and [¹²⁵I]protein A. Synthesis of

^{35}S -labeled precursor proteins was carried out in a reticulocyte lysate system (Hurt et al., 1984a); hemin was omitted because it did not stimulate translation in our system. For import of urea-denatured precursors, we took advantage of the observation that a fraction of the precursor molecules were associated with the ribosomal pellet after centrifugation: the translation mixture was incubated with 100 mM glycerol plus 50 U/mL *Escherichia coli* glycerokinase for 5 min at 30 °C, followed by centrifugation in a Beckman Airfuge for 10 min at $150,000 \times g$, and the resulting pellet was solubilized in 8 M urea, 20 mM Tris-HCl (pH 7.0), and 10 mM dithiothreitol. Unless otherwise indicated, both nondenatured and urea-denatured precursors were diluted 20-fold into the final import mixture. "Standard import buffer" consisted of 0.6 M sorbitol (deionized), 50 mM K^+ -4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.0), 50 mM KCl, 10 mM MgCl_2 , 2 mM KH_2PO_4 , 2.5 mM EDTA, 5 mM methionine, and 1 mg/mL fatty acid-free bovine serum albumin; mitochondria were added to a final concentration of 0.25 mg/mL protein. Import reactions were shielded from light to prevent photooxidation. Where indicated, hemin was added from a 1 mM stock solution in dimethyl sulfoxide; we found that solutions prepared by dissolving hemin at high pH and then neutralizing were inhibitory to import. Unless otherwise stated, mitochondria were reisolated after import by centrifuging 5 min at $12,000 \times g$ and were resuspended in standard import buffer supplemented with 25 μM FCCP. Where indicated, the mitochondria were then treated with proteinase K and/or converted to mitoplasts. Finally, the organelles were reisolated once again and precipitated with trichloroacetic acid. In some experiments two different precursors were co-imported into the same mitochondria, but in other cases, separate reactions were performed in parallel because we found that incomplete import of cytochrome b_2 resulted in proteolytic products that comigrated with some of the other radiolabeled species.

DNA constructions

The starting plasmid for all manipulations was pCL1, which contains the gene for the yeast cytochrome b_2 precursor (Guiard, 1985) cloned as an EcoRI-HindIII fragment into pSP65 (Promega). Cytochrome b_2 - $\Delta 14$ was created by digesting pCL1 with ClaI and BamHI, filling in with Klenow enzyme, and religating; this procedure deleted the codons corresponding to amino acids 152–165 of the precursor. To make $\text{pb}_2(1-167)$ -DHFR, the DHFR gene from pDS5/2 (Stueber et al., 1984) was excised with BamHI and HindIII and subcloned into pCL1 cut with the same enzymes; the resulting construct encodes the first 167 amino acids of the cytochrome b_2 precursor fused to DHFR by the two-residue linker GI. The $\text{pb}_2(1-185)$ -DHFR was created by cutting plasmid pDS5/2-1-PCOXIV-DHFR (Hurt et al., 1984b) with

XhoI, filling in with Klenow, then excising the DHFR gene with HindIII and subcloning it into pCL1 cut with EcoRV and HindIII; the resulting construct encodes the first 185 amino acids of the cytochrome b_2 precursor fused to DHFR by the five-residue linker SRSGL. Construction of the plasmid-encoding $\text{pb}_2(1-180)$ has been described (Brunt et al., 1992). The fusion protein Su9-DHFR was kindly provided by Walter Neupert; this protein comprises the first 69 residues of the precursor of subunit 9 of the *Neurospora crassa* mitochondrial ATPase fused to DHFR (Pfanner et al., 1987b).

ATP depletion procedures

Mitochondria are referred to as "fully energized" if ATP was present on both sides of the inner membrane; the term "ATP-depleted mitochondria" indicates that the matrix was depleted of ATP, regardless of whether ATP was present outside the inner membrane. The methods used were those described by Wachter et al. (1992), with minor modifications. We found that oligomycin nonspecifically inhibited the import of several precursor proteins, including cytochrome c_1 and $\text{pb}_2(1-167)$ -DHFR; this effect could be measured by inactivating the mitochondrial ATP synthetase with efrapeptin, energizing the mitochondria with ATP plus NADH, and then adding various amounts of oligomycin. For the experiments described here, we used a combination of 2 $\mu\text{g}/\text{mL}$ efrapeptin and 5 $\mu\text{g}/\text{mL}$ oligomycin, which efficiently blocked ATP synthesis but caused only a weak nonspecific inhibition of import. All ATP depletion experiments included control reactions in which the mitochondria were fully energized.

"No ATP" or "None"

ATP was depleted both inside and outside the inner membrane as in Wachter et al. (1992), except that precursors were treated with hexokinase as described below under "ATP in."

"ATP out"

Matrix ATP was specifically depleted as described by Wachter et al. (1992). In the experiment of Figure 2A, α -ketoglutarate was subsequently used to regenerate matrix ATP by substrate-level phosphorylation (Tzagoloff, 1982). Measurements of intramitochondrial ATP levels (see below) confirmed that addition of α -ketoglutarate caused efficient generation of matrix ATP, even in the absence of an electrochemical potential across the inner membrane.

"ATP in"

ATP outside the inner membrane (also referred to as external ATP) was selectively depleted. We used the following improved procedure. After translation of the precursor protein, the reticulocyte lysate was depleted of ATP by incubating 5 min at 30 °C with 50 U/mL yeast

hexokinase (Sigma) plus 20 mM 2-deoxyglucose. Ribosomes were removed by centrifugation for 10 min at $150,000 \times g$, and the spun lysate was diluted 1:20 into the import mixture. The mitochondria (0.25 mg/mL in standard import buffer) were preincubated with 20 μ g/mL carboxyatractyloside plus 2 mM NADH for 5 min on ice. To deplete external ATP, *E. coli* glycerokinase (10 U/mL; Sigma) and glycerol (10 mM) were then added, and the mixture was incubated 3 min at 30 °C followed by 3 min at the import temperature before addition of the hexokinase-treated precursor. For the control with fully energized mitochondria, glycerokinase was omitted and 1 mM ATP plus an ATP-regenerating system (5 mM creatine phosphate, 50 μ g/mL rabbit muscle creatine kinase) were added instead. This method is superior to the one described by Wachter et al. (1992) because the same precursor preparation is used for both the positive control (ATP in + out) and the test reaction (ATP in), so that the experiment specifically measures the reversible inhibition of import caused by depletion of external ATP.

Determination of intramitochondrial ATP levels

ATP was measured with the firefly luciferase assay (Lundin, 1982) using the ATP monitoring kit from Bio-Orbit (Turku, Finland). One vial of the lyophilized monitoring reagent was dissolved in 5 mL of the supplied Tris-acetate buffer. The solution was divided into 0.25-mL aliquots, "aged" 2 h at room temperature (Leach & Webster, 1986), frozen in liquid nitrogen, and stored at -80°C . Assay mixtures contained 20 μ L monitoring reagent in a final volume of 200 μ L Tris-acetate buffer. The light signal was recorded with a Packard 2200CA liquid scintillation counter in noncoincidence mode using the ^3H channel and single photon counting. After addition of the ATP-containing sample, the solution was mixed by gentle pipetting, placed in the scintillation counter, and counted twice for 1 min each time. The second measurement was more accurate because a background signal from photoluminescence was eliminated. This assay was linear for final ATP concentrations of 10^{-11} – 10^{-8} M (approx. 4×10^3 to 4×10^6 cpm above a background of 2×10^4 cpm with no added ATP).

The total amount of ATP present in a solution of mitochondria was measured as follows: 3.0 μ L 70% perchloric acid was added while mixing to 37.5 μ g mitochondria in 150 μ L standard import buffer. This treatment inactivated endogenous mitochondrial ATPases as well as *E. coli* glycerokinase. After 10 min on ice the precipitated protein was removed by centrifuging 5 min at $12,000 \times g$. To 120 μ L of the supernatant was added 17 μ L 2 N KOH while mixing, and after 3 min on ice, the precipitated KClO_4 was removed by centrifuging 5 min at $12,000 \times g$. The pH of the resulting solution was between 7 and 8. Of this neutralized supernatant, 1–2 μ L was added per 200 μ L ATP assay. The results were calibrated

by adding known amounts of ATP to samples of import buffer lacking mitochondria and then precipitating these samples and analyzing them as described above. Protein-bound ATP was measured by dissolving the mitochondria with 0.2% Triton X-100 and leaving the mixture on ice for 5 min in the presence of glycerokinase and glycerol; control experiments confirmed that this treatment completely hydrolyzed 1 μ M exogenous ATP added after the detergent. The ATP that remained inaccessible to glycerokinase in the presence of detergent could be degraded by acid denaturing the proteins, neutralizing the mixture, and then adding fresh glycerokinase. The measured amounts of protein-bound ATP were essentially identical if the lysed mitochondria were incubated with glycerokinase for 5 min at 30 °C.

In order to determine the ATP concentration in the matrix, the volume of matrix per milligram mitochondrial protein was estimated as follows. Assuming that mitochondria are 15% protein by weight, 1 mg protein corresponds to a total mitochondrial mass of 6.7 mg, which will occupy a volume of slightly less than 6.7 μ L in aqueous medium. If the matrix accounts for about 60% of the volume of an average mitochondrion, the combined matrix volume for 1 mg mitochondrial protein will be approx. 4 μ L.

Quantitation of folding and assembly of radiolabeled proteins

Folding of the heme-binding domain was measured by quantifying the amount of the 10.5-kDa core fragment recovered after trypsin treatment. The N-terminal residue of the core fragment is presumably Leu 4 of the mature protein, although the tryptic cleavage site is not known with certainty because the starting material in earlier studies was a modified cytochrome b_2 lacking the first seven residues (Guiard et al., 1975). The C-terminal residue of the core fragment is Lys 103 of the mature protein (Guiard & Lederer, 1976); the fragment from pb $_2$ (1–180) is slightly smaller because this precursor terminates after residue 100 of mature cytochrome b_2 . The core fragment should contain 2 of the 10 methionine residues in pre-cytochrome b_2 or pb $_2$ (1–185)-DHFR, and 2 of the 3 methionines in pb $_2$ (1–180). There are two methionines in the destabilized heme-binding domain of cytochrome b_2 - $\Delta 14$, and one methionine in the truncated heme-binding domain of pb $_2$ (1–167)-DHFR.

Trypsin digestion of fully assembled cytochrome b_2 resulted in the formation of several characteristic fragments in addition to the 10.5-kDa core (Gervais & Tegoni, 1980). The fragment designated β' was resistant to high levels of trypsin and was used to quantify assembly of cytochrome b_2 . Generation of β' involves cleavage in a protease-sensitive loop within the flavin domain (Ghrir & Lederer, 1981; Xia & Mathews, 1990). Assuming that all of the lysine residues in this loop are good substrates for trypsin,

β' should contain three of the nine methionine residues in mature cytochrome b_2 .

Differential scanning calorimetry

A Microcal MC-2D instrument fitted with an EM Electronics N2a nanovolt preamplifier was used at a scan rate of 60 °C/h. The heme-binding domain of cytochrome b_2 (Brunt et al., 1992) was prepared as a 2.6-mg/mL solution in 10 mM Tris-HCl (pH 7.5). Sample and reference solutions were degassed for approx. 1 min under vacuum with gentle stirring before being loaded and were held under 2–3 atm N_2 pressure during the scan to inhibit degassing and bubble formation at higher temperatures. The data were normalized by subtraction of values obtained with buffer alone and were converted to units of specific heat capacity by assuming a molecular weight of 10,450 for the cytochrome b_2 core and a cell volume calculated as described previously (Johnson et al., 1992).

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