Successive Translocation into and out of the Mitochondrial Matrix: Targeting of Proteins to the Intermembrane Space by a Bipartite Signal Peptide

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Summary

We investigated the import and sorting pathways of cytochrome b_2 and cytochrome c_1, which are functionally located in the intermembrane space of mitochondria. Both proteins are synthesized on cytoplasmic ribosomes as larger precursors and are processed in mitochondria in two steps upon import. The precursors are first translocated across both mitochondrial membranes via contact sites into the matrix. Processing by the matrix peptidase leads to intermediate-sized forms, which are subsequently redirected across the inner membrane. The second proteolytic processing occurs in the intermembrane space. We conclude that the hydrophobic stretches in the presequences of the intermediate-sized forms do not stop transfer across the inner membrane, but rather act as transport signals to direct export from the matrix into the intermembrane space.

Introduction

Transport of proteins into mitochondria can be subdivided into several distinct steps (for reviews see Hay et al., 1984; Harmey and Neupert, 1985; Pfanner and Neupert, 1987): Nuclear-coded mitochondrial proteins are synthesized on free polysomes as precursors, most of which have amino-terminal peptide extensions, and are released into cytosolic pools (Hallermayer et al., 1977; Schatz, 1979). Precursors are located to mitochondria via targeting signals contained either in the presequences or in some cases in the mature parts of the precursors (Hurt et al., 1984a; 1984b; Horwich et al., 1985; Pfanner et al., 1987b). They bind to specific receptors on the surface of mitochondria (Zwizinski et al., 1984). Insertion into and translocation across the inner mitochondrial membrane are dependent on the mitochondrial membrane potential (Schleyer et al., 1982; Gasser et al., 1982a; Kolanski et al., 1982; Pfanner and Neupert, 1985) and in addition requires nucleoside triphosphates (Pfanner and Neupert, 1986; Pfanner et al., 1987c; Chen and Douglas, 1987; Eilers et al., 1987; Hartl et al., 1987a); translocation occurs at contact sites between outer and inner membranes (Schleyer and Neupert, 1985; Hartl et al., 1986; Schwaiger et al., 1987; Hartl et al., 1987a; Pfanner et al., 1987a). Precursors are proteolytically processed by the metal-dependent processing peptidase located in the mitochondrial matrix (Böhni et al., 1980; Miura et al., 1982; Conboy et al., 1982;

McAda and Douglas, 1982; Schmidt et al., 1984). Finally, most of the imported proteins are assembled into supra-molecular complexes (Lewin and Norman, 1983; Schmidt et al., 1983; Dowhan et al., 1985; Hartl et al., 1986).

This overall sequence of events, however, does not describe the individual reactions responsible for the intramitochondrial sorting of precursor proteins to the different submitochondrial compartments. Proteins of the intermembrane space or of the outer surface of the inner membrane reaching into the intermembrane space seem to follow the most complex sorting and assembly pathways. Two-step proteolytic processing has been found to be a common feature for some of these proteins including cytochrome b_2 (Daum et al., 1982; Gasser et al., 1982b), cytochrome c peroxidase (Kaput et al., 1982), cytochrome c_1 (Gasser et al., 1982b; Ohashi et al., 1982; Teintze et al., 1982), and the Rieske Fe/S protein of complex III (Hartl et al., 1986). In each case the first proteolytic processing step is performed by the matrix peptidase; that is, precursors have to be translocated either completely or at least partially across the inner membrane.

In the present report we describe distinct steps in the import and sorting pathways of cytochromes b_2 and c_1. Cytochrome b_2 is a soluble component of the intermembrane space of yeast mitochondria (Daum et al., 1982). Cytochrome c_1 is anchored to the inner membrane but reaches into the intermembrane space with a large hydrophilic portion (Li et al., 1981). Both precursors are imported via translocation contact sites (Schleyer and Neupert, 1985; Hartl et al., 1987a; Pfanner et al., 1987a). They are made with large presequences of 80 amino acids in the case of cytochrome b_2 (Guiard, 1985) and of 61 and 70 residues in case of yeast and Neurospora cytochrome c_1, respectively (Sadler et al., 1984; Römisch et al., 1987). These prepieces have two distinguishable domains. Their amino-terminal parts exhibit the features of typical matrix targeting signals (Hurt and van Loon, 1986), while their carboxy-terminal parts contain a hydrophobic stretch of approximately 20 amino acid residues. This region has been suggested to function as a "stop-transfer" sequence prohibiting complete translocation of the precursor across the inner mitochondrial membrane (Guiard, 1985; van Loon et al., 1986; van Loon and Schatz, 1987; van Loon et al., 1987). A similar mechanism has been proposed for the precursor to cytochrome c peroxidase (Kaput et al., 1982).

The experiments presented here suggest that the precursors of cytochromes b_2 and c_1 are first translocated across both membranes into the matrix of mitochondria in a membrane potential–dependent reaction. Following cleavage by the processing peptidase, the resulting intermediate-sized species are directed across the inner membrane in the opposite direction. The second processing, which takes place at the outer surface of the inner membrane, leads to the formation of the mature proteins.

We conclude that the hydrophobic segments in the carboxy-terminal parts of the presequences do not stop
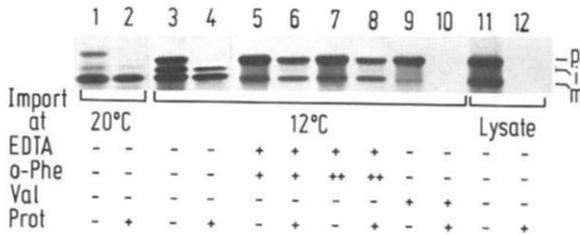
Figure 1. Import of Cytochrome b₂ into Isolated Mitochondria

A postribosomal supernatant of a reticulocyte lysate containing radiolabeled p-b₂ was supplemented with unlabeled methionine and sucrose and diluted 1:5 with BSA buffer (see Experimental Procedures). It was divided into 200 μl aliquots, and each was made 2 mM NADH and 1.5 mM ATP (added from a 100 mM stock solution in water, neutralized to pH 7.0 with 1 N KOH prior to use). Where indicated, reactions were brought to 10 mM EDTA, 1 mM (+), and 2 mM (++) o-phenanthroline (o-Phe), respectively, or to 1 μM valinomycin (Val). To each reaction, mitochondria were added to a final concentration of 0.5 mg/ml, and incubation was for 30 min at 20°C (lanes 1 and 2) or 12°C (lanes 3–10). Afterward mitochondria were reisolated and resuspended in 200 μl of SEOM buffer (0.25 M sucrose, 5 mM EDTA, 1 mM o-phenanthroline, and 10 mM MOPS [pH 7.2]) and divided into two portions. One portion was left on ice for 30 min (lanes 1, 3, 5, 7, and 9); the other was treated with proteinase K (Prot) at 15 μg/ml, final concentration (lanes 2, 4, 6, 8, and 10). Then all reactions received PMSF to 1 mM, and mitochondria were reisolated. The mitochondrial pellets were washed once with SEOM buffer and finally dissociated in SDS-containing sample buffer for electrophoresis. As a control, one aliquot of reticulocyte lysate was diluted 1:5 with SEOM and divided into halves, one of which was treated with proteinase K. Both reactions were precipitated with TCA. Of the amount of lysate used in the import reactions, 50% was applied in lanes 11 and 12. A fluorograph of the dried gel is shown. The positions of p-b₂ (p), i-b₂ (i), and m-b₂ (m) are indicated.

transport of the precursors across the inner mitochondrial membrane, but function as signals to direct the reverse translocation of the intermediate-sized forms from the mitochondrial matrix across the inner membrane. We suggest that the mechanism of this export from the mitochondrial matrix shares a common origin with the mechanism of protein export from the cytoplasm into the periplasmic space in prokaryotes.

Results

Upon Inhibition of the Processing Peptidase, Precursor to Cytochrome b₂ Is Imported into a Protease-Protected Position by Isolated Yeast Mitochondria

Precursor to cytochrome b₂ (p-b₂) was synthesized from the cloned DNA by coupled transcription–translation in rabbit reticulocyte lysate in the presence of [³⁵S]methionine. For import of the precursor, reticulocyte lysate and isolated mitochondria were combined and incubated at different temperatures. At 20°C, precursor was imported into mitochondria and processed to mature-sized cytochrome b₂ (m-b₂) (Figure 1, lane 1); this m-b₂ was in a position protected from externally added proteinase K (Figure 1, lane 2). Under these conditions, only small amounts of precursor were present on the surface of mitochondria. When import was performed at 12°C, in addition to m-b₂,

considerable amounts of precursor and intermediate-sized b₂ (i-b₂) accumulated (Figure 1, lanes 3 and 4). Precursor was digested by protease, whereas 50%–60% of i-b₂ and some 90% of m-b₂ were protected. We have recently shown that at lower temperatures (7°C–12°C) i-b₂ spanning both outer and inner membranes at translocation contact sites accumulated (Pfanner et al., 1987a). Protease-sensitive i-b₂ formed at 12°C represented such a contact site intermediate.

When the chelators EDTA and o-phenathroline were added in combination to the import reaction at 12°C to inhibit the processing peptidase in the matrix (Schmidt et al., 1984), formation of m-b₂ was decreased and large amounts of the precursor were associated with mitochondria (Figure 1, lanes 5–8). Most of this precursor was resistant to added protease; thus, it was at least translocated across the outer membrane. In contrast to results with the Rieske Fe/S protein in Neurospora mitochondria (Hartl et al., 1986), processing of p-b₂ in yeast mitochondria could be inhibited only partially. The amount of i-b₂ relative to m-b₂ is decreased because formation of i-b₂ is much slowed by the presence of metal chelators, whereas its conversion to m-b₂ is not affected.

Import of cytochrome b₂ was dependent on the membrane potential across the inner mitochondrial membrane. When the membrane potential was dissipated by adding the potassium ionophore valinomycin (in the presence of 80 mM KCl outside), precursor and small amounts of "pseudomature" b₂ were associated with the surface of mitochondria; both were completely degraded by added proteinase K (Figure 1, lanes 9 and 10). The same sensitivity to added protease was found for precursor and pseudomature b₂ in the reticulocyte lysate, indicating that adequate concentrations of protease were applied in the protection assays (Figure 1, lanes 11 and 12). Formation of pseudomature b₂ most likely resulted from internal initiation of translation at the methionine in position +6 of the mature sequence (Guiard, 1985).

Imported Precursor of Cytochrome b₂ Is Located in the Matrix of Mitochondria

The various forms of imported cytochrome b₂ were analyzed for their extractability by 0.1 M sodium carbonate (data not shown) (for method see Pfanner et al., 1987a). m-b₂ was fully extractable, as was to be expected for a soluble intermembrane space protein. Also, precursor that was in a protease-protected position and both protease-sensitive and protease-protected i-b₂ were completely solubilized by treatment at alkaline pH. Apparently p-b₂ and i-b₂ either were not membrane-integrated species or were present in the inner membrane in a hydrophilic environment accessible to aqueous perturbants.

To examine further the submitochondrial arrangement of the various forms of cytochrome b₂, we analyzed their solubility in buffers of increasing ionic strength (Figure 2). Imported m-b₂ and preexistent unlabeled m-b₂ (measured as lactate ferricyanide reductase) were largely soluble without addition of salt to the extraction buffer. A salt concentration of 0.2 M was sufficient to extract 60%–70% of imported p-b₂ and about 40% of i-b₂. Higher salt con-
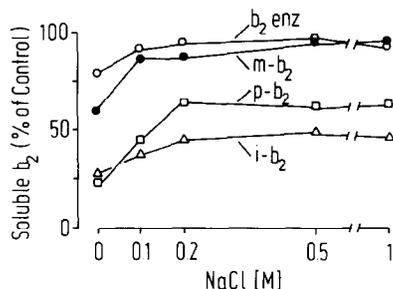
Figure 2. Extractability of the Various Forms of Cytochrome b_2 with Salt

Mitochondria were reisolated from import reactions and resuspended at a protein concentration of 0.1 mg/ml in SEOM buffer containing 0–1 M NaCl. Samples were sonicated at 0°C–4°C (Branson sonifier with top-end microtip; setting 3, pulsed, 40% duty, three 1 min pulses with two 1 min cooling intervals) and divided into two portions. One was kept on ice for determination of recoveries (total); the other was separated into pellets and supernatants by centrifugation for 1 hr at 165,000 × g. Pellets were resuspended in buffer containing the respective salt concentration and brought to the same volume as supernatants. Aliquots corresponding to 25 μg of untreated mitochondria were taken for determination of cytochrome b_2 enzyme activity. All samples were made 0.0125% in deoxycholate, and after 5 min on ice, TCA was added to a final concentration of 10%. Incubation on ice was continued for 30 min, and precipitates were collected by centrifugation (15 min, 48,000 × g). They were washed once in acetone and finally dissociated in SDS-containing buffer. Fluorographs of dried gels were analyzed by densitometry. Data are expressed as the percentages of total cytochrome b_2 recovered in the supernatants. Recoveries of total cytochrome b_2 in supernatants and pellets varied between 85% and 98%. Open circles indicate enzyme activity of preexisting b_2 (b_2 enz); closed circles, m-b_2 imported at 20°C; open squares, protease-protected p-b_2 accumulated at 12°C in the presence of EDTA and o-phenanthroline; open triangles, protease-protected i-b_2 accumulated at 12°C. Experimental conditions for import as in Figure 1.

Figure 3. Release of Imported p-b_2 and i-b_2 from Mitochondria by Treatment with Digitonin

In one reaction import was performed at 12°C to accumulate i-b_2 and m-b_2. In another import reaction EDTA and o-phenanthroline were added to accumulate p-b_2 (for experimental details see Figure 1). Mitochondria were reisolated and resuspended in SEOM buffer. Both reactions were combined. After treatment with proteinase K, mitochondria were again reisolated and resuspended at a protein concentration of 10 mg/ml in SEOMK buffer (SEOM buffer containing 200 mM KCl, 0.1 mM PMSF, and 0.5 mg/ml protease inhibitor from N. crassa). The reaction was divided into nine aliquots. Digitonin was added to concentrations of 0%–0.3%, and incubation was for 1 min at 0°C in a final volume of 30 μl and at a protein concentration of 5 mg/ml. Afterward reactions were diluted 5-fold with SEOMK and separated into pellets and supernatants. Pellets were resuspended in SEOMK to the same volume as the supernatants. Aliquots corresponding to 50 μg of untreated mitochondria were precipitated with TCA. The remaining parts of the samples were brought to 1% Genapol (Hoechst) and used for determination of marker enzymes. TCA precipitates were separated by electrophoresis, and fluorographs were evaluated by densitometry. Enzyme activities and amounts of cytochrome b_2 in supernatants (A) and pellets (B) are expressed as percentages of the total activities. b_2-enz, enzymatic b_2 measured as lactate ferricyanide reductase activity; Fum, fumarase.

centrations did not lead to further release of these species into the soluble fraction.

Most of imported p-b_2 and approximately half of i-b_2 were water-soluble and were therefore not integrated into the inner membrane. Approximately 30% of p-b_2 and 50% of i-b_2, however, behaved differently in that they were tightly associated with the inner membrane.

Soluble precursor and intermediate could thus be located in either the intermembrane space or the matrix of mitochondria. To decide which is the correct location, mitochondria that had imported precursor in the presence of chelators and mitochondria that had imported precursor in the absence of chelators were mixed (see Figure 1), incubated with proteinase K to digest material accessible to external protease, and treated with increasing concentrations of digitonin in the presence of 0.2 M salt. The contents of intermembrane space (marker lactate-ferricyanide reductase) and matrix (marker fumarase) were successively released (Figure 3A). Imported m-b_2 cofractionated exactly with preexisting cytochrome b_2 and was released at low concentrations of digitonin between 0.025% and 0.075%, while fumarase was still found in the pellet fractions (Figure 3B). Imported precursor and intermediate soluble at 0.2 M salt (see Figure 2) were released from mitochondria in parallel with fumarase. It therefore appears

that two-thirds of imported p-b_2 and about one-half of i-b_2 are soluble proteins located in the matrix of mitochondria.

This result was further substantiated when mitochondria containing accumulated precursor or intermediate were fractionated by osmotic shock treatment (Daum et al., 1982; van Loon et al., 1986). Most of the soluble intermembrane space content (preexisting cytochrome b_2) was released after incubation in 0–0.1 M sorbitol, whereas only 10%–15% of fumarase was set free (data not shown). After osmotic shock, the different incubations were brought to isoosmolarity and treated with proteinase K. Preexisting b_2 and imported m-b_2 were completely digested in shocked mitochondria (Figure 4A, lanes 3 and 4); however, more than 60% of the precursor was still resistant to externally added protease. In contrast, only

Figure 5. Processing of Imported p-b$_2$ to m-b$_2$

Mitochondria were incubated in reticulocyte lysates containing radiolabeled p-b$_2$ for 30 min at 12°C (experimental conditions of import as in Figure 1). The first group of reactions (lanes 1–3) contained valinomycin (+Val) to block the membrane potential across the inner membrane. To the second and third groups of reactions (lanes 4–6 and lanes 7–9) EDTA and o-phenanthroline (+EDTA/o-Phe) was added to inhibit the processing peptidase in the matrix. After import mitochondria were reisolated and resuspended in sucrose/MOPS buffer containing 0.5 mM EDTA and 0.5 mM o-phenanthroline to maintain the block of the peptidase. The second group of reactions (lanes 4–6) was treated with proteinase K (+Prot) to remove p-b$_2$ either bound to the surface of mitochondria or accumulated in contact sites. Prior to a chase period for 15 min at 20°C, valinomycin and MnCl$_2$ (1 µM and 1.5 mM, final concentrations, respectively) were added when indicated. Subsequently, mitochondria were reisolated and analyzed by electrophoresis and fluorography. The positions of p-b$_2$ (p), i-b$_2$ (i), and m-b$_2$ (m) are indicated.

Figure 4. Submitochondrial Localization of Imported b$_2$ by Osmotic Shock Treatment of Mitochondria

(A) Accessibility to protease of the various forms of b$_2$ in mitoplasts. Mitochondria that had accumulated p-b$_2$ and p-Fe/S and mitochondria that had accumulated i-b$_2$ and m-b$_2$ were combined (for experimental details see Figure 3). They were divided into five aliquots of 200 µg each and incubated for 15 min at 0°C in MOPS buffer containing 5 mM EDTA, 1 mM o-phenanthroline, and 0–0.3 M sorbitol (final concentrations) (see Experimental Procedures). Then the reactions were adjusted to 0.6 M sorbitol. Half of each reaction was separated into pellets and supernatants and used for the determination of lactate ferricyanide reductase and fumarase (data not shown). The other halves were treated with proteinase K (+Prot) and aliquots were used for enzyme determination (lower panel). The residual parts of the samples were TCA-precipitated and analyzed by electrophoresis and fluorography (lanes 1–5). The reaction shown in lane 5 was made 1% Triton X-100 (+TX) prior to treatment with proteinase K. The amount of protease-protected p-Fe/S was quantified by densitometry (lower panel). Data are expressed as percentages of total p-Fe/S and enzyme activities, respectively, protease-protected in controls (0.3 M sorbitol). The positions of p-b$_2$ (p), i-b$_2$ (i), and m-b$_2$ (m) are indicated.
(B) Extractability by salt of p-b$_2$ accumulated in mitoplasts. Mitochondria that had accumulated p-b$_2$ were incubated in 0.3 M sorbitol (a) or 0.05 M sorbitol (b) (experimental conditions as in [A]). After protease treatment, mitochondria and mitoplasts, respectively, were reisolated and subjected to salt extraction in SEOM buffer containing 200 mM NaCl (experimental conditions as in Figure 2). After electrophoresis and fluorography, p-b$_2$ in pellets (P) and supernatants (S) was quantified by densitometry. Data are expressed as percentages of total p-b$_2$ protease-protected in intact mitochondria. M, mitochondria (0.3 M sorbitol); MP, mitoplasts (0.05 M sorbitol).

30%–40% of i-b$_2$ remained protease-protected in osmotically shocked mitochondria. As a sensitive indicator for protease access to the matrix, precursor of Rieske Fe/S protein was accumulated in the matrix in addition to cytochrome b$_2$ (Hartl et al., 1986; van Loon and Schatz, 1987). This p-Fe/S was protease-protected in mitochondria with ruptured outer membrane to about the same extent as was p-b$_2$. The protease protection of p-b$_2$, i-b$_2$,

and p-Fe/S in mitoplasts was completely abolished after mild sonication or addition of Triton X-100.

p-b$_2$ protected against protease in mitoplasts was tested for its solubility (Figure 4B, columns b). This fraction of the precursor was found to be completely soluble at a salt concentration as low as 0.2 M NaCl, in contrast to total imported precursor (see above; Figure 4B, columns a).

These data show that p-b$_2$ was translocated across both mitochondrial membranes and was present as a water-soluble species in the matrix. A fraction of total imported precursor (approximately 35%) was tightly membrane- associated and was located on the outer surface of the inner membrane in mitoplasts. Likewise, about 60% of i-b$_2$ accumulated at 12°C was exposed to the outer face of the inner membrane, the other 40% being soluble in the matrix.

Precursor and Intermediate-Sized Form of Cytochrome b$_2$ Accumulated in the Matrix Are on the Correct Import Pathway

Precursor was imported into mitochondria at 12°C in the presence of EDTA and o-phenanthroline, i.e., under conditions where the matrix peptidase was partially inhibited (Figure 5, lanes 4–9). Half of the sample was treated with proteinase K to remove precursor bound to the surface of mitochondria (Figure 5, lanes 4–6). Mitochondria were then reisolated and resuspended in fresh sucrose/MOPS buffer to which different additions were made. When EDTA and o-phenanthroline were added as during import, the block of the matrix peptidase was maintained (Figure 5, lanes 4 and 7). When Mn^{2+} ions were added and the reactions were warmed up to 20°C, most of p-b$_2$ became processed to m-b$_2$ (Figure 5, lanes 5, 6, 8, and 9). Also, in the case in which treatment with protease after import had

A
Import
12°C

1 2 3

p
i
m

Chase
20°C

i
m

B 100
%Activity retained
50
0

Fum
b_2

0   .075   .1
% Digitonin
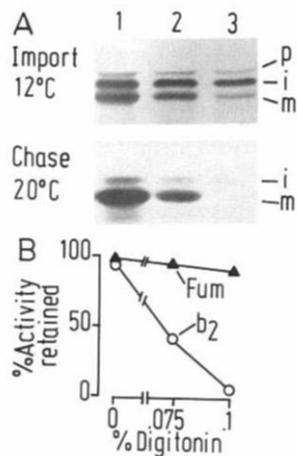
Figure 6. Transport of i-b_2 from the Matrix across the Inner Membrane

Mitochondria that had accumulated i-b_2 and m-b_2 at 12°C (for experimental details see Figure 1) were reisolated and resuspended in sucrose/MOPS buffer containing 80 mM KCl and 1 μM valinomycin. The sample was halved. The first half was kept on ice, while the second was incubated for 15 min at 20°C (Chase). Mitochondria were reisolated from both samples, and digitonin fractionation was performed (experimental details as in Figure 3). Concentrations of digitonin applied were 0%, 0.075%, and 0.1% (corresponding to lanes 1–3).
(A) Reactions were treated with proteinase K, TCA-precipitated, and analyzed by electrophoresis and fluorography. The positions of p-b_2 (p), i-b_2 (i), and m-b_2 (m) are indicated.
(B) Aliquots were taken prior to protease treatment and separated into pellets and supernatants. Marker enzymes lactate ferricyanide reductase (b_2) and fumarase (Fum) were determined. Data are expressed as enzyme activities retained in pellets. Results were not influenced by whether reactions were submitted to the chase at 20°C or were kept on ice. Therefore only one set of data is shown.

been omitted, very little precursor remained unprocessed. Most likely part of this precursor, which had not yet been in a protease-protected position (Figure 5, lane 4 and 7; see Figure 1), was spanning both outer and inner membranes at contact sites. In agreement with this, p-b_2 was chased to m-b_2 with the same efficiency when the membrane potential across the inner membrane had been dissipated by valinomycin (plus potassium) (Figure 5, lanes 6 and 9). Thus precursor located in contact sites was already past the membrane potential–dependent step of the import pathway. As a control for the effective inhibition of import, valinomycin was included during the import reaction (Figure 5, lanes 1–3). In this case no processing of p-b_2 was observed during a subsequent chase period.

To substantiate further the view that matrix-localized p-b_2 and i-b_2 are on the native import and sorting pathway, a second type of experiment was performed. Import was carried out at 12°C, resulting in the formation of both i-b_2 and m-b_2 (Figure 6). Mitochondria were reisolated and resuspended in fresh import buffer. Valinomycin was included to dissipate the membrane potential. One-half of the reaction was then incubated at 20°C, while the other half was kept at 12°C. Mitochondria were treated with low concentrations of digitonin to open the intermembrane space selectively. Then the resulting mitoplasts were treated with proteinase K. With a concentration of 0.1%

digitonin the outer membrane barrier was sufficiently disrupted to completely set free preexisting cytochrome b_2 (determined enzymatically), while most of fumarase activity was retained. Imported m-b_2 was digested (Figure 6, lane 3). In agreement with the results shown in Figure 4, about 40% of i-b_2 imported at 12°C was protected against protease in mitoplasts (Figure 6, lane 3). During the chase period at 20°C, most of this i-b_2 was processed to protease-sensitive m-b_2 (Figure 6, lanes 1–3). The small amount of i-b_2 that remained unprocessed also became accessible to protease.

From these data we conclude that i-b_2 accumulated in the matrix at low temperature became redirected across the inner membrane. At the outer surface of the inner membrane, processing of i-b_2 results in the formation of m-b_2, which is then soluble in the intermembrane space. It seems possible that p-b_2 can also be retranslocated from the matrix to the intermembrane space, although with lower efficiency than i-b_2. The fraction of imported p-b_2 that was resistant to extraction with salt and became protease-sensitive in mitoplasts probably had already reached the matrix and was retranslocated across the inner membrane (Figure 4B).

Precursor of Cytochrome c_1 Is Imported into a Protease-Protected Position by Isolated Neurospora Mitochondria

In contrast to the results stated above, it was proposed recently that the hydrophobic segment in the presequence of cytochrome c_1 stops transfer of the precursor across the inner membrane (van Loon et al., 1986). In particular, precursor of a fusion protein between the presequence of cytochrome c_1 and dihydrofolate reductase was never detected in a protease-protected position in mitoplasts upon import in vitro (van Loon and Schatz, 1987). To resolve the problem as to whether or not cytochromes b_2 and c_1 follow different pathways, we have investigated the import of cytochrome c_1 into Neurospora mitochondria.

Radioactively labeled precursor to cytochrome c_1 from Neurospora crassa was synthesized from the cloned cDNA by coupled transcription–translation and imported into isolated mitochondria. Appropriate experimental conditions were chosen to accumulate distinct intermediates on the import pathway (Figure 7). In the presence of the chelators EDTA and o-phenanthroline, unprocessed precursor (p-c_1) was translocated into a position within mitochondria protected against added proteinase K (Figure 7, lanes 1 and 2). As with import of cytochrome b_2, inhibition of the first processing step was more efficient when import was carried out at lower temperature (Figure 7, lane 1 versus lane 2, 8°C versus 25°C). The second proteolytic processing from intermediate-sized c_1 (i-c_1) to mature c_1 (m-c_1) was strongly dependent on the presence of NADH. When NADH was omitted from the import reaction, i-c_1 was accumulated, which was also in a protease-protected location (Figure 7, lane 3). Presence of NADH led to the formation of m-c_1 (Figure 7, lane 4). When formation of a mitochondrial membrane potential was inhibited by a combination of antimycin A and oligomycin, import was completely abolished (Figure 7, lane 5).
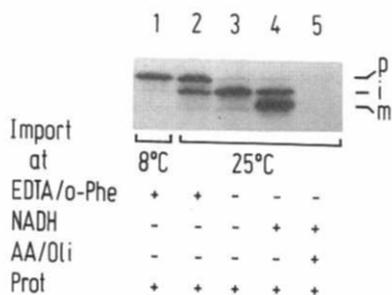
Figure 7. Import of Cytochrome c_1 into Isolated Mitochondria

A postribosomal supernatant of a reticulocyte lysate containing radio-labeled p-c_1 was supplemented with unlabeled methionine and sucrose and diluted 1:10 with BSA buffer. Where indicated it was brought to 2 mM NADH, 5 mM EDTA, 0.1 mM o-phenanthroline (EDTA/o-Phe), or 10 μM and 40 μM antimycin A and oligomycin (AA/Oli), respectively. To four 100 μl aliquots, mitochondria of N. crassa were added to a concentration of 0.5 mg/ml. Incubation was for 30 min at 25°C. Then mitochondria were reisolated and resuspended in sucrose/MOPS buffer containing chelators as above. Treatment with proteinase K (Prot) (15 μg/ml, final concentration) was performed for 30 min on ice. Mitochondria were reisolated, dissociated in SDS-containing buffer, and analyzed by electrophoresis and fluorography. The positions of p-c_1 (p), i-c_1 (i), and m-c_1 (m) are indicated.

Translocation of the Precursor of Cytochrome c_1 across the Inner Membrane Is Not Prevented by the Hydrophobic Segment in the Presequence

p-c_1 and p-Fe/S were imported into mitochondria at 8°C in the presence of EDTA and o-phenanthroline. After import, mitochondria were reisolated and treated with increasing concentrations of digitonin and a fixed concentration of proteinase K (Figure 8). Precursor to c_1 exhibited the same degree of protease resistance as p-Fe/S. When the intermembrane space of mitochondria was opened by low concentrations of digitonin (as monitored by the release of adenylate kinase; data not shown), both precursors were still protected against added protease. They were only degraded when the digitonin concentration was raised to cause the release of the matrix marker fumarase (Figure 8, reaction 1; also see Figure 3). Specific fragments of p-c_1 due to partial digestion were not detectable.

If p-c_1 was first imported at 8°C as described above and the incubation mixture was then warmed to 25°C for 15 min in the presence of Mn^{2+} to reactivate the processing peptidase, i-c_1 was formed. When a membrane potential was established during the chase, this i-c_1 was digested by added protease once the outer membrane barrier had been disrupted by low concentrations of digitonin (Figure 8, reaction 2). However, i-c_1 was still largely protected against added protease in mitoplasts if prior to reactivation of the processing peptidase the membrane potential across the inner membrane had been abolished by a combination of antimycin A and oligomycin (Figure 8, reaction 3).

We interpret these results to indicate that p-c_1 was first translocated across both mitochondrial membranes. After the first processing step, i-c_1 was translocated in the reverse direction across the inner membrane to where the major part of the protein was exposed to the intermem-
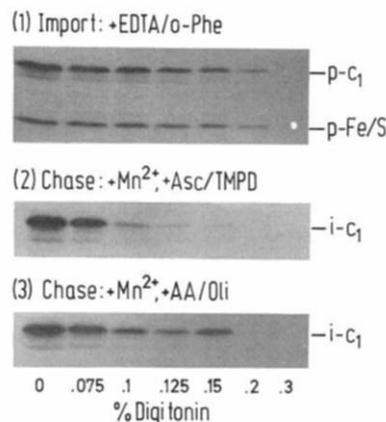
Figure 8. Submitochondrial Localization of Imported p-c_1 and i-c_1

p-c_1 and p-Fe/S were imported at 8°C in the presence of EDTA and o-phenanthroline (experimental conditions as in Figure 7). The sample was divided into three reactions. Mitochondria were reisolated and resuspended in BSA buffer. Reaction (1) was made 5 mM EDTA and 0.1 mM o-phenanthroline to maintain the block of the peptidase. Reaction (2) received 8 mM ascorbate (added from a 10-fold concentrated stock solution, neutralized to pH 7.0 with KOH) and 0.2 mM TMPD. Reaction (3) was made 10 μM antimycin A and 40 μM oligomycin. Then reactions (2) and (3) were brought to 1 mM $MnCl_2$ to release the block of the peptidase and incubated for 15 min at 25°C (Chase). Reaction (1) remained on ice. Mitochondria were again reisolated from all three reactions and subjected to fractionation with digitonin (experimental conditions as in Figures 3 and 6). Afterward each fraction was divided into halves. One half was seperated into pellets and supernatants for determination of marker enzymes (data not shown); the other was treated with proteinase K (15 μg/ml, final concentration). Mitochondria were reisolated and analyzed by electrophoresis and fluorography.

brane space. Export of i-c_1 from the matrix across the inner membrane apparently was dependent on an energized inner membrane. So far it is unknown whether this behavior, which differs from the retranslocation of i-b_2, is due to differences between the mitochondria of the two species or is specific for cytochrome c_1.

The possibility that the differential protease digestibility of p-c_1 and i-c_1 could be an artifact due to a higher endogenous protease resistance of p-c_1 was carefully controlled for. After addition of 1% Triton X-100 to the protection assay, both species were completely degraded by the amount of protease routinely used (data not shown). We also titrated the protease resistance of imported p-c_1 and i-c_1 after disruption of the mitochondrial membranes by mild sonication (Figure 9). Already very low concentrations of proteinase K (5 μg/ml) were sufficient to digest 70% of imported p-c_1 and i-c_1 (Figure 9A). The residual part remained protease-protected also at higher concentrations of proteinase K. It was, however, completely digested when protease was present during sonication (Figure 9B). Sonication is known to result in the formation of inner membrane vesicles of both orientations.

Due to technical reasons, we were unable to decide whether p-c_1 was also accumulated in the matrix as a soluble species. Less than 10% of imported p-c_1 was extractable in the presence of high salt concentrations (0.5

A

B
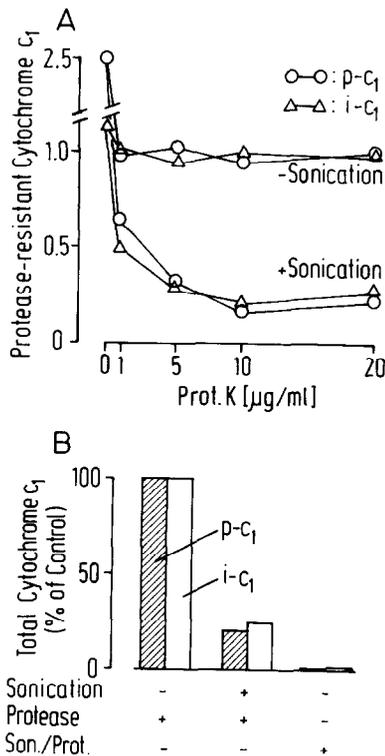
Figure 9. Titration of Endogenous Protease Resistance of p-c_1 and i-c_1

Import of cytochrome c_1 was performed in two separate reactions. In the first one p-c_1 was accumulated in the presence of EDTA and o-phenanthroline; in the second i-c_1 was generated by omitting NADH during import (experimental conditions as in Figure 7). Mitochondria were reisolated and resuspended in sucrose/MOPS buffer containing EDTA and o-phenanthroline. Then both reactions were combined. Half of the resulting sample was sonicated as described in Figure 2. The other half was left on ice. Both halves were divided into aliquots corresponding to 50 μg of untreated mitochondria.

(A) Five aliquots of sonicated mitochondria and five aliquots of intact mitochondria were treated with the indicated amounts of proteinase K (Prot. K) for 30 min on ice. Protease action was blocked by adding PMSF, and cytochrome c_1 was immunoprecipitated (see Experimental Procedures). Precipitates were separated by electrophoresis, and fluorographs of dried gels were evaluated by densitometry. The amount of cytochrome c_1 protected against proteinase K in intact mitochondria was set to 1.

(B) Three aliquots of intact mitochondria were subjected to sonication and protease treatment (15 μg/ml, final concentration) as indicated. In one aliquot protease was added prior to sonication (Son./Prot.). In this case protease treatment was continued after sonication for 25 min at 0°C. Cytochrome c_1 was immunoprecipitated, and the amounts of p-c_1 (hatched columns) and i-c_1 (open columns) were quantified. Data are expressed as percentages of total cytochrome c_1 protease-protected in intact mitochondria.

M NaCl). To control for the significance of this result, we incubated reticulocyte lysate containing newly synthesized (soluble) p-c_1 with sonicated mitochondrial membranes in the presence of different concentrations of salt. Upon centrifugation, more than 90% of this p-c_1 was recovered in the pellet. The same result was obtained with membranes that had been treated with 0.1 M Na_2CO_3 at pH 11.5 according to Fujiki et al. (1982) (data not shown).
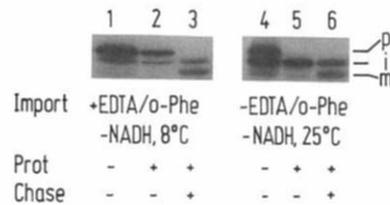
Figure 10. Processing of Imported p-c_1 and i-c_1 to m-c_1

p-c_1 was imported into mitochondria in two groups of reactions (experimental conditions as in Figure 7). The first contained EDTA and o-phenanthroline to accumulate imported p-c_1 (lanes 1–3). In the second group of reactions import was performed in the absence of chelators. NADH was omitted to accumulate i-c_1 (lanes 4–6). Mitochondria were reisolated and resuspended in sucrose/MOPS buffer containing chelators (lanes 1–3) or in sucrose/MOPS buffer alone (lanes 4–6). Two reactions of each group were treated with proteinase K (lanes 2, 3, 5, and 6). Prior to a chase period for 30 min at 25°C, reactions shown in lanes 3 and 6 received a postribosomal supernatant of untranslated reticulocyte lysate and BSA buffer to 25% and 50% of the total volume, respectively, and both reactions were brought to 1 mM NADH. In addition, reaction shown in lane 3 received 1 mM $MnCl_2$ (final concentration). Reactions shown in lanes 1, 2, 4, and 5 remained for 30 min on ice. Then mitochondria were reisolated from all reactions and analyzed by electrophoresis and fluorography.

Precursor to Cytochrome c_1 Imported into the Matrix Is on the Correct Import Pathway

Precursor was imported at low temperature into a protease-protected location in the mitochondrial matrix (Figure 10, lanes 1 and 2). Mitochondria were reisolated and resuspended in fresh import buffer allowing for the reactivation of the processing peptidase. During a subsequent chase period, Mn^{2+}, NADH, and untranslated reticulocyte lysate were added and the reaction warmed up to 25°C (Figure 10, lane 3). Under these conditions, most of the accumulated p-c_1 was processed to m-c_1. Thus, precursor translocated across both mitochondrial membranes is an authentic intermediate on the native assembly pathway. In addition to NADH, the presence of a factor contained in reticulocyte lysate was found to be necessary for efficient processing of i-c_1 to m-c_1. Intermediate c_1 accumulated in the absence of NADH (Figure 10, lanes 4 and 5) could be processed to m-c_1 with the same efficiency as p-c_1 (Figure 10, lane 6). These data suggest that p-c_1 follows a route into the intermembrane space very similar to p-b_2.

Discussion

The following import and sorting pathway of cytochrome b_2 is suggested. Precursor first binds specifically to receptors on the outer surface of mitochondria (Riezmann et al., 1983). Then complete translocation across outer and inner membranes takes place at contact sites (Hartl et al., 1987a; Pfanner et al., 1987a). This step is dependent on the membrane potential across the inner membrane. Under conditions where the activity of the processing peptidase is inhibited, precursor accumulates in the matrix. After the first proteolytic processing, i-b_2 is redirected back across the inner membrane. This step appears to be

independent of the membrane potential. The second proteolytic event then occurs at the outer surface of the inner membrane, thereby generating m-b_2 soluble in the intermembrane space.

Import and sorting of cytochrome c_1 follows the same general pathway. Also, in this case precursor is first translocated across both mitochondrial membranes. Due to the property of newly synthesized p-c_1 to attach unspecifically to mitochondrial membranes, it was impossible to decide whether p-c_1 is also accumulated in the matrix as a soluble species. It is possible that imported p-c_1 interacts with the inner surface of the inner membrane either via the hydrophobic segment in the presequence or via the potential hydrophobic anchor near the carboxyl terminus of the molecule (Römisch et al., 1987). In any case, the precursor is translocated across the inner membrane since it was found protected against externally added protease in mitoplasts but was completely digested following mild mechanical disruption of mitochondria. i-c_1 is generated by the processing peptidase in the matrix and is transported back across the inner membrane. Processing at the outer surface of the inner membrane yields m-c_1, which remains anchored to the inner membrane, probably via the hydrophobic stretch of 15 amino acids near the C-terminus (Römisch et al., 1987).

Transfer of p-b_2 and p-c_1 across the inner membrane during import was not stopped by the hydrophobic stretch of about 20 amino acid residues, contained in the carboxy-terminal part of the presequences (positions 53 to 73 in b_2 and positions 43 to 62 in c_1; Guiard, 1985; Römisch et al., 1987). We have recently shown for several different precursors (including cytochrome b_2) that transport across both mitochondrial membranes at contact sites occurs through a hydrophilic environment (possibly a proteinaceous channel) (Pfanner et al., 1987a). A potential hydrophobic stop-transfer sequence (Kaput et al., 1982; Hurt and van Loon, 1986), however, could only be effective by strong protein–lipid interaction; this does not seem to occur during transport of proteins from the cytoplasm across the mitochondrial membranes. Our experimental data rather suggest that the N-terminal prepieces of intermediate-sized b_2 and c_1 contain the targeting information for export of the protein across the inner membrane. In both cases the intermediate-sized species were retranslocated. Removal of the prepieces of the intermediates occurs at the outer surface of the inner membrane by an as yet uncharacterized protease(s) (see also Pratje and Guiard, 1986). Membrane translocation is mechanistically independent of processing.

Precursor of the Rieske Fe/S of the cytochrome bc_1 complex (complex III) is also first translocated into the matrix of mitochondria in the course of import (Hartl et al., 1986; Hartl et al., 1987b). In this case, however, retranslocation from the matrix across the inner membrane follows a different mechanism. The precursor of the Fe/S protein lacks a hydrophobic segment in the presequence. Processing to the mature size already takes place inside the inner membrane, i.e., targeting information for retranslocation and assembly resides in the mature part of the protein (Hartl and Neupert, unpublished data). Interestingly,

a bacterial Fe/S protein that was found to be synthesized without a cleavable hydrophobic signal sequence is translocated into the periplasmic space and may thus follow a transport mechanism similar to the mitochondrial Rieske Fe/S protein (Voordouw and Brenner, 1985).

Recently it was reported that the presequence of yeast cytochrome c_1 when fused to mouse dihydrofolate reductase directed the "passenger" protein into the mitochondrial intermembrane space (van Loon et al., 1986; van Loon and Schatz, 1987; van Loon et al., 1987). As a mechanism for intramitochondrial sorting, the authors proposed that the hydrophobic segment in the carboxy-terminal part of the presequence could act as a stop-transfer sequence, preventing complete translocation of the precursor across the inner membrane. A construct lacking this part of the presequence was imported into the matrix. These experiments, however, did not exclude the possibility that transient import of the fusion protein carrying the authentic presequence of c_1 into the matrix had also occurred, since intermediates on the import pathway were not resolved. More recently, van Loon and Schatz (1987) presented data showing that the fusion between the full presequence of cytochrome c_1 and dihydrofolate reductase after import in vitro was not found in a protease-protected position in mitoplasts when the matrix peptidase was blocked by chelators. (Import of authentic p-c_1 was not tested.)

In the light of our data, these results may be explained as follows. It was not shown that the accumulated precursor of the construct was on the native assembly pathway (e.g., by releasing the block of the matrix peptidase to demonstrate processing; see Figures 8 and 10). Furthermore, it was not excluded that import into the matrix took place but the uncleaved precursor was retranslocated across the inner membrane. When we performed import of p-b_2 and p-c_1 at 20°C instead of at 12°C or 8°C, we found significantly less protease-protected precursor in mitoplasts (the intactness of mitoplasts was not influenced by the temperature during incubation). The fraction of total p-b_2 imported at 12°C that was protease-sensitive in mitoplasts probably represented precursor that was retranslocated from the matrix across the inner membrane. It has been demonstrated that precursor proteins could be translocated across the membrane of the endoplasmic reticulum or across the bacterial plasma membrane even when unrelated sequences had been fused to the amino terminus of the signal peptide (Perara and Lingappa, 1985; Simon et al., 1987; Rottier et al., 1987; Talmadge et al., 1981; Hayashi et al., 1985). Finally, it is quite possible that the fusion protein between the presequence of cytochrome c_1 and dihydrofolate reductase behaved differently compared with the authentic precursor. In fact, the fusion protein was never cleaved to the mature form, but remained at the intermediate stage. It may well be that the hydrophobic sequences in precursors of cytochromes b_2 and c_1 act properly only in conjunction with the authentic mature protein; when placed in front of an unrelated protein they could lead to aberrant behavior of the precursor proteins in the course of sorting.

The characteristics of the retranslocation of the inter-

mediate-sized species of cytochromes b_2 and c_1 across the inner membrane are reminiscent of those of protein transport across the plasma membrane in prokaryotes. In bacteria, proteins destined for secretion are synthesized with amino-terminal signal peptides that typically carry several positively charged residues at the amino-terminal end followed by a hydrophobic stretch of 14 to 20 residues (Duffaud et al., 1985). In fact, the intermediate prepieces of cytochromes b_2 and c_1 have a similar structure. The N-termini of the intermediate forms of cytochromes b_2 and c_1 have not yet been determined. However, comparison with a number of known cleavage sites of the matrix peptidase (Schmidt et al., 1984; Hartl et al., 1986) suggests that the intermediate forms begin 21 and 15 amino acid residues, respectively, before the hydrophobic segments (Guiard, 1985; Sadler et al., 1984; Römisch et al., 1987). The exact mechanism of transport of i-b_2 and i-c_1 across the inner membrane remains to be determined. The intermediates may assume a membrane-spanning arrangement after retranslocation, leaving the mature part exposed to the intermembrane space. The extractability of i-b_2 by sodium carbonate would then indicate that the membrane-associated segment is shielded by proteins (possibly of the retranslocation apparatus) from direct lipid contact.

Based on the hypothesis of the endosymbiotic origin of mitochondria, we suggest that transport of proteins from the matrix across the inner membrane follows a mechanism already developed in the prokaryotic ancestor of mitochondria. The cytochrome bc_1 complex of the photosynthetic bacterium Rhodopseudomonas sphaeroides contains a cytochrome c_1 homologous in structure, function, and topology to the mitochondrial c_1 (Gabellini et al., 1985; Hauska et al., 1983). It is made in the cytoplasm of the bacterium with a 21 amino acid presequence that is similar to the prepiece of the intermediate form of mitochondrial cytochrome c_1 (Gabellini and Sebald, 1986; Sadler et al., 1984; Römisch et al., 1987). In the bacterium the precursor is translocated to the periplasmic side of the photosynthetic membrane and processed to the mature subunit. We propose that this assembly pathway has been conserved during evolution and is still followed in mitochondria with regard to export from the matrix (corresponding to the bacterial cytoplasm) across the inner membrane (corresponding to the bacterial plasma membrane). The same principle seems to be followed in intrachloroplast sorting for transport from the stroma across the thylakoid membrane (Smeekens et al., 1986). Cytochrome f, the equivalent of cytochrome c_1 in the thylakoid cytochrome b_6f complex (Hauska et al., 1983), is made in the chloroplast stroma with an amino-terminal presequence of 35 amino acids that again exhibits typical features of bacterial signal sequences and similarity to the intermediate prepiece of mitochondrial c_1 (Alt and Herrmann, 1984). We propose that mitochondrial precursors with a bipartite presequence destined for the intermembrane space are first imported into the matrix. After processing by the matrix peptidase, the intermediate enters the ancestral assembly pathway.

Experimental Procedures

Isolation of Mitochondria

Wild-type Saccharomyces cerevisiae (D 273–10B) was grown on 2% lactate and mitochondria were isolated essentially according to Daum et al. (1982). For preparation of spheroplasts, cells were incubated with Zymolyase-20T (Seikagaku Kogyo Co.) at 5 mg per g wet weight of cells for 30 min at 30°C. Homogenization of spheroplasts was carried out by 10 strokes in a Dounce homogenizer in a medium containing 0.6 M mannitol, 0.5 % BSA (Sigma), 1 mM PMSF, and 10 mM Tris (pH 7.4). Mitochondria were finally resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH [pH 7.2]) at a protein concentration of 5 mg/ml. Growth of N. crassa (wild type 74A) and isolation of mitochondria were performed as previously described (Schleyer et al., 1982; Schwaiger et al., 1987).

Import of Precursor Proteins In Vitro

p-b_2, p-c_1, and p-Fe/S were synthesized by coupled transcription–translation (Stueber et al., 1984; Krieg and Melton, 1984; Pelham and Jackson, 1976). For cytochrome b_2, the genomic clone described previously was used (Guiard, 1985). For cytochrome c_1 and the Fe/S protein, a full-length cDNA was isolated from an N. crassa library constructed according to Gubler and Hoffmann (1983) and cloned into pGEM-3 (Hartl et al., 1987a; Rassow and Neupert, unpublished data). Postribosomal supernatants of reticulocyte lysates were prepared and supplemented as published (Zimmermann and Neupert, 1980). Reticulocyte lysates lacking labeled mitochondrial precursor proteins were prepared according to Pfanner and Neupert (1985). Import mixtures usually contained 10%–20% reticulocyte lysate in BSA buffer (3% BSA, 220 mM sucrose, 80 mM KCl, and 10 mM MOPS/KOH [pH 7.2]) and 2 mM NADH. In addition, 1.5 mM ATP was included for import of cytochrome b_2. Mitochondria were added to a concentration of 0.5 mg per ml of import reaction. Inhibition of the matrix-localized processing peptidase, inhibition of the mitochondrial membrane potential, and protease treatment of mitochondria after import were carried out as recently described (Schmidt et al., 1984; Hartl et al., 1986). Control reactions received the same volume of inhibitor-free solutions.

Subfractionation of Mitochondria

Alkaline treatment of mitochondria (Fujiki et al., 1982) and analysis of solubility of imported cytochrome b_2 (Hartl et al., 1986) were carried out as published. Digitonin fractionation was carried out in the presence of 100 mM KCl (cytochrome c_1) or 200 mM KCl (cytochrome b_2) as described previously (Hartl et al., 1986). Osmotic shock treatment of mitochondria followed the method of Daum et al. (1982). Mitochondria were reisolated from the import reactions by centrifugation and resuspended at a concentration of 1 mg/ml in 10 mM MOPS (pH 7.2) containing 0–0.6 M sorbitol. Incubation was for 15 min at 0°C. Then the reactions were brought to 0.6 M sorbitol by addition of 1.2 M sorbitol, adjusting the protein concentration to 0.5 mg/ml. Aliquots corresponding to 25 μg of untreated mitochondria were treated with proteinase K (15 μg/ml, final concentration) for 30 min on ice or separated into pellets and supernatants by centrifugation for 20 min at 48,000 × g.

Miscellaneous

The following procedures were performed according to published methods: enzyme assays of cytochrome b_2 (Appleby and Morton, 1959), adenylate kinase, and fumarase (Schmidt et al., 1984); immunoprecipitation of cytochrome b_2, cytochrome c_1, and the Fe/S protein (Schleyer et al., 1982; Hartl et al., 1986); protein determination (Bradford, 1976); electrophoresis on 10% (cytochrome b_2) and 17% (cytochrome c_1 and Fe/S protein) polyacrylamide gels (Laemmli, 1970); fluorography and quantitation of fluorographs by densitometry (Hartl et al., 1986).

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