Cytochrome Systems

Molecular Biology and Bioenergetics

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IMPORT OF CYTOCHROMES b_2 AND c_1 INTO MITOCHONDRIA IS DEPENDENT ON BOTH MEMBRANE POTENTIAL AND NUCLEOSIDE TRIPHOSPHATES

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SUMMARY

Import of precursors of cytochromes b_2 and c_1 into mitochondria requires a mitochondrial membrane potential. We show here that in addition to $\Delta\Psi$, nucleoside triphosphates (NTPs) are necessary for protein translocation. At low concentrations of NTPs, intermediate-sized cytochrome b_2 was accumulated spanning the outer and inner membranes at contact sites. For complete translocation into mitochondria, higher concentrations of NTPs were necessary. We conclude that different levels of NTPs are required for distinct steps in the import pathway.

INTRODUCTION

Most mitochondrial proteins are coded for by the nucleus and are synthesized as precursors on cytoplasmic polysomes. Transport of proteins into mitochondria can be subdivided into several steps (for reviews see: Harmey and Neupert, 1985; Pfanner and Neupert, 1987; Hartl et al., 1987): (i) specific interaction with receptors on the mitochondrial surface; (ii) transport into mitochondria at translocation contact sites between outer and inner membranes; (iii) processing of precursors having N-terminal extensions by the processing peptidase in the matrix; (iv) additional modifications, including covalent or non-covalent attachment of cofactors or, in certain cases, a second proteolytic processing step; and (v) assembly into active supramolecular protein complexes.

Transport into or translocation across the inner membrane is dependent on an energized inner membrane (Hallermayer and Neupert, 1976; Nelson and Schatz, 1979; Zimmermann et al., 1981; Schleyer et al., 1982; Gasser et al., 1982; Kolanski et al., 1982). Energy is required in the form of the electrical component $\Delta\Psi$ of total protonmotive force (Pfanner and Neupert, 1985). For long, however, it could not be decided whether high energy phosphate compounds, such as ATP, are necessary in addition to $\Delta\Psi$. Indeed, this was recently shown for the import of the ß subunit of F_0F_1-ATPase (F_1ß) (Pfanner and Neupert, 1986), the ADP/ATP-translocator of the inner membrane, and fusion proteins between F_0F_1-ATPase subunit 9 and dihydrofolate reductase (Pfanner et al., 1987).

In the present report we investigated whether the import of

cytochromes b_2 and c_1 was dependent on NTPs. Cytochrome b_2 is a soluble component of the intermembrane space (Daum et al., 1982a). Cytochrome c_1 is anchored to the inner membrane but contains a large hydrophilic domain which protrudes into the intermembrane space (Li et al., 1981). Compared to ß subunit of F_oF_1-ATPase their import and sorting pathways are more complex in that both precursor proteins are proteolytically processed in two steps (Daum et al., 1982b; Ohashi et al., 1982; Teintze et al., 1982). The first processing step is performed by the matrix peptidase, i.e. precursors have to be translocated either completely or at least partially across the inner membrane. The second processing event occurs at the outer surface of the inner membrane by a so far uncharacterized protease(s) (Pratje and Guiard, 1986). On addition both proteins have to acquire heme, which is covalently attached in case of cytochrome c_1.

Selective and independent manipulation of NTP levels and the membrane potential showed that import of cytochromes b_2 and c_1 required both NTPs and $\Delta\Psi$. Interestingly, at low levels of NTPs precursors were only partially translocated into mitochondria: they accumulated in translocation contact sites (Schleyer and Neupert, 1985).

MATERIALS AND METHODS

Growth of Neurospora crassa (wild type 74A) (Schleyer et al., 1982) and isolation of mitochondria by Percoll (Pharmacia) density gradient centrifugation was done as described (Hartl et al., 1986). Yeast cells of wild type Saccharomyces cerevisiae (D273-10B) were grown on 2% lactate and mitochondria were isolated according to Daum et al. (1982a). Mitochondria were finally suspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) at a protein concentration of 2.5 mg/ml.

Precursor proteins were synthesized by coupled transcription/translation. For cytochrome c_1, a full length cDNA was isolated from a N. crassa library and cloned into pGEM4. For cytochrome b_2, the genomic clone described previously (Guiard, 1985) was used. Transcription/translation of the cloned sequences followed the methods of Krieg and Melton (1984) and Stueber et al. (1984), respectively. Postribosomal supernatants were prepared and supplemented as published (Schleyer et al., 1982).

Labelled reticulocyte lysates and isolated mitochondria were treated with apyrase (Sigma, grade VIII) essentially as described before (Pfanner and Neupert, 1986). Afterwards reticulocyte lysates were cooled to 0°C and diluted with BSA buffer (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, 3% (w/v) BSA, pH 7.2). Antimycin A, oligomycin or valinomycin (8 µM, 20 µM and 1 µM, respectively) were added from 100-fold concentrated stock solutions in ethanol when indicated. Mixtures for import into N. crassa mitochondria contained 8 mM potassium ascorbate and 0.2 mM N,N,N',N'-tetramethylphenylenediamine (TMPD) as an energy source, whereas mixtures for yeast mitochondria included 20 mM potassium succinate. Then mitochondria (50 µg of protein) were added. In order to supplement NTPs, either ATP or GTP (8 mM final concentration) were added from 200 mM stock solutions in water. For neutralization sufficient amounts of 1 M MOPS/NaOH, pH 7.2, were included. Incubation was for 30 min at 25°C in a total volume of 100 µl. Mitochondria were then reisolated by centrifugation (15 min 27,000 x g), resuspended in SEM buffer and treated with proteinase K (15 µg/ml final concentration) as described (Hartl et al., 1986). Reisolated mitochondria were lysed in SDS sample buffer. SDS polyacrylamide electrophoresis and fluorography were carried out according to published methods (Laemmli, 1970; Hartl et al., 1986).

RESULTS

Import of cytochromes b_2 and c_1 requires nucleoside triphosphates

Reticulocyte lysates containing the ^{35}S-methionine labelled precursor of cytochrome b_2 and freshly isolated yeast mitochondria were pretreated with different concentrations of apyrase, an adenosine 5'-triphosphatase and adenosine 5'-diphosphatase which caused rapid depletion of endogenous ATP and ADP. Mitochondria and reticulocyte lysate were mixed in the presence of succinate (as a respiratory substrate) and oligomycin (which inhibits the F_0F_1-ATPase, Wikstrom and Krab, 1982). The latter was included to prevent reduction of the mitochondrial membrane potential by ATPase activity. Following incubation for 30 min at 25°C, the samples were treated with proteinase K to digest cytochrome b_2 that had not been imported into mitochondria. Then mitochondria were reisolated and dissolved in SDS-containing buffer for subsequent

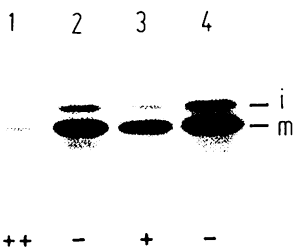

Fig. 1. Import of cytochrome b_2 into yeast mitochondria is inhibited by apyrase treatment.
Reticulocyte lysates containing labelled precursor of cytochrome b_2 were incubated for 15 min at 30°C and 15 min at 25°C with apyrase: reactions 1 and 3 received 1 and 0.25 U/ml, respectively; reactions 2 and 4 received corresponding amounts of inactivated apyrase. Mitochondria were added to the lysate in presence of succinate and oligomycin. Incubation for import was for 30 min at 25°C. Afterwards the samples were cooled to 0°C and diluted 1:2 with SEM. Proteinase K treatment was then performed (30 min at 0°C). Protease activity was stopped by adding PMSF to 1 mM. Then mitochondria were reisolated by centrifugation and dissociated in SDS-containing buffer. The samples were analyzed by electrophoresis and fluorography. Abbreviations: i, intermediate; m, mature cytochrome b_2.

electrophoresis and fluorography. A fluorograph of the dried gel is shown in Fig.1. Control samples received an apyrase preparation which had been inactivated by heating to 95°C for 10 min. In these reactions, cytochrome b_2 precursor was imported into a protease protected location and processed to the mature form. Besides mature cytochrome b_2, a small quantity of the intermediate sized form (about 20% of total) was observed which was also protected against externally added protease (Fig.1, lanes 2 and 4). When lysate and mitochondria had been pretreated with apyrase, import was clearly diminished (Fig.1, lanes 1 and 3). Apyrase per se did reduce neither the protease resistance of endogenous mitochondrial proteins nor the amount of precursor proteins in the reticulocyte lysate (Pfanner and Neupert, 1986; Pfanner et al., 1987). We conclude that pretreatment with apyrase causes inhibition of import of cytochrome b_2 into mitochondria.

To test whether the apyrase effect was due to depletion of NTPs, experiments were performed where ATP or GTP were included during import (Fig.2).

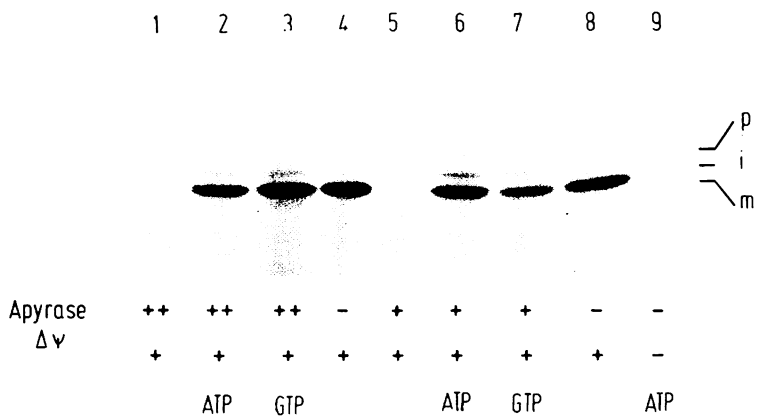

Fig. 2. Import of cytochrome b_2 from apyrase treated reticulocyte lysate is restored by addition of ATP or GTP.
Reticulocyte lysates and mitochondria were incubated with the following concentrations of apyrase: reactions 1- 3, 4 U/ml; reactions 5-7, 1 U/ml; reaction 4 received 4 U inactivated apyrase/ml and reactions 8 and 9 1 U/ml. Import and protease treatment was performed as described in legend to Fig.1, except that in reactions 2,6 and 9, 8 mM ATP and in reactions 3 and 7, 8 mM GTP were added during import. In reaction 9, 1 µM valinomycin was added prior to ATP. A fluorograph of the dried gel is shown.
Abbreviations as in Fig.1. p, precursor of cytochrome b_2.

Under these conditions the import of cytochrome b_2 could be fully restored (Fig.2, lanes 2,3 and 6,7). This import, however, was completely abolished when valinomycin plus potassium ions were added to destroy the membrane potential across the inner membrane (Fig.2, lane 9). In this case, addition of ATP did not restore import.

The same result was obtained with import of cytochrome c_1 into mitochondria of N. crassa. Experimental conditions were essentially as described for import of cytochrome b_2, except that ascorbate/TMPD was used to establish a membrane potential and NADH was included, which is required for the second maturation step of cytochrome c_1 (Schleyer and Neupert, 1985, see accompanying article by Nicholson et al.). A fluorograph corresponding to the experimental design presented in Fig.2 was quantified by densitometry (Fig.3).

Again apyrase treatment drastically reduced import and processing (Fig.3, lanes 1,5) which could be restored by addition of ATP or GTP (Fig.3, lanes 2,3,6,7). Import was completely blocked after inhibition of the membrane potential with antimycin A and oligomycin and could not be restored by including ATP in the reaction (Fig.3, lane 9). Consequently, the presence of NTPs and a membrane potential are two separate requirements. NTPs cannot substitute for the requirement of $\Delta\psi$. At present,

however, it is unknown which form of high energy phosphate is actually needed since nucleoside phosphate kinases present in mitochondria can lead to formation of various nucleoside triphosphates.

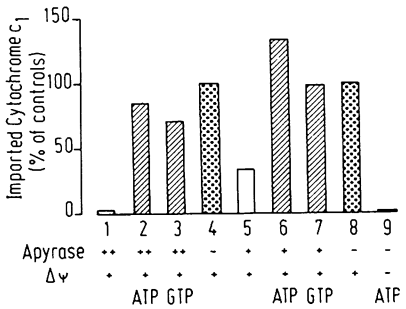

Fig. 3. Import of cytochrome c_1 is inhibited by apyrase treatment and restored by ATP or GTP.
The experimental design was essentially as described in the legend to Fig.2, except that mitochondria from N. crassa were used and import was performed in the presence of ascorbate/TMPD and NADH instead of succinate. Antimycin A was included in addition to oligomycin. Formation of mature cytochrome c_1 was quantified by densitometry of the fluorograph. The amount of mature c_1 formed in controls (inactivated apyrase) was set at 100%.

Intermediate-sized cytochrome b_2 spanning outer and inner membranes at contact sites can be accumulated at low levels of NTPs

For precursors of ß subunit of F_0F_1-ATPase, cytochrome c_1 and the Fe/S-protein of complex III, it has been previously shown that import into mitochondria performed at lower temperatures results in the formation of so called "contact site intermediates" (Schleyer and Neupert, 1985; Hartl et al., 1986). Such an intermediate is characterized as follows: the N-terminal presequence of the precursor has been translocated across both membranes in a $\Delta\psi$ dependent manner and is cleaved off by the processing peptidase in the matrix. A large part of the polypeptide, however,is still outside the mitochondrion where it can be digested by externally added protease. It follows that these features can be only fulfilled at regions where outer and inner membranes are close enough together to be spanned by a single polypeptide chain.

Experiments with apyrase pretreatment indicated that after depletion of ATP the formation of intermediate-sized cytochrome b_2 was not reduced to the same extent as was processing to the mature form. In contrast to mature b_2, more than 85% of which were protease-resistent, this intermediate was largely sensitive to externally added protease. This effect could be clearly demonstrated when apyrase concentrations were titrated over a wider range (0 to 100 U apyrase/ml of lysate or mitochondrial suspension). After import, one half of each reaction was treated with proteinase K. Following electrophoresis and fluorography the

amounts of intermediate-sized b_2 formed were quantified by densitometry (Fig. 4).

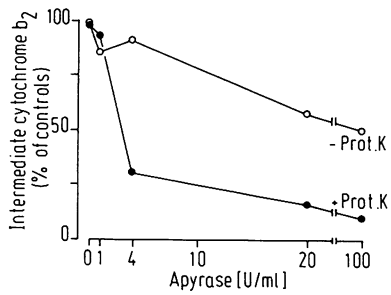

Fig 4. Intermediate sized cytochrome b_2 is accumulated in contact sites at low levels of NTPs.
Reticulocyte lysates and mitochondria were incubated with apyrase (0-100 U/ml). Controls received corresponding amounts of inactivated apyrase. Import and protease treatment were performed as described in the legend to Fig.1, except that only half of each sample received protease. Formation of intermediate cytochrome b_2 was quantified by densitometry of the fluorograph. The amount of intermediate b_2 formed in controls (inactivated apyrase) was set at 100%.

At apyrase concentrations between 4 and 20 U/ml, the formation of intermediate b_2 was reduced by 10 to 40% compared to controls that had received inactivated apyrase. Most of this intermediate sized b_2, however, was digested by externally added proteinase K thus fulfilling the criteria for a contact site intermediate described above. Only with very low concentrations of apyrase was intermediate-sized b_2 protected against externally added protease. Within a narrow range of 1 to 4 U apyrase/ml, protease protection decreased from 95 to 30% indicating that only above a distinct level of NTPs import into protease protected position did take place. When the membrane potential was dissipated, no processing to intermediate b_2 was observed. We conclude that partial translocation of the precursor can occur at low levels of NTPs; this step depends on the potential across the inner membrane. For complete translocation, however, higher levels of NTPs are required.

DISCUSSION

The import of precursors of cytochromes b_2 and c_1 into mitochondria needs NTPs in addition to $\Delta\psi$. At low levels of NTPs, a translocational intermediate of cytochrome b_2 could be accumulated in contact sites.

Owing to the presence of nucleoside phosphate kinases it is unclear so far which form of high energy phosphate compound (eg. ATP or GTP) is the

direct energy source. Non-hydrolyzable ATP-analogues could not restore import after depletion of NTPs with apyrase (Pfanner and Neupert, 1986) indicating that the mechanism of action of NTPs involves the hydrolysis of high energy phosphate bonds. The dependence of protein import into mitochondria on NTPs seems to be a general phenomenon. Similar effects have been recently observed with the precursors of ß subunit of F_0F_1-ATPase (Pfanner and Neupert, 1986) and the ADP/ATP-translocator (Pfanner et al., 1987). Interestingly, the insertion of porin into the outer membrane (which is independent of $\Delta\Psi$) also seems to need NTPs (Kleene et al., in preparation).

What could be the role of NTPs? Previous results had already suggested that NTPs modify the conformation of cytosolic mitochondrial precursor proteins (Pfanner et al., 1987). Eilers and Schatz (1986) have demonstrated that lack of tertiary structure is a prerequisite for protein import into mitochondria. Taking the resistance against digestion by protease as a measure for the degree of tertiary structure it could be shown that the presence of NTPs results in the unfolding of precursor molecules (Pfanner et al., 1987) thus rendering them competent for translocation across the mitochondrial membranes. In the present study, $\Delta\Psi$ dependent partial translocation of the precursor of cytochrome b_2 was possible at very low concentrations of NTPs resulting in an intermediate reaching into the matrix with its aminoterminus but having a large part of the molecule still outside the mitochondrion. Complete translocation into a protease protected position was then achieved by adding ATP or GTP and was independent of $\Delta\Psi$ (Hartl and Neupert, unpublished). These findings are consistent with the idea that the membrane potential is only necessary for the translocation of the aminoterminal part of the precursor across the inner membrane. It is assumed to exert an electrophoretic effect on the positive charges contained in the presequence (Pfanner and Neupert, 1985; Roise et al., 1986). Complete translocation of the precursor across both membranes is only possible when the polypeptide is kept in an unfolded state, the energy source for the unfolding reaction being NTPs. Specific binding of the precursor to receptors on the surface of mitochondria, insertion into and partial translocation across the mitochondrial membranes is possible at very low levels of NTPs; thus, it seems likely that the presequence folds independently of the mature part of the precursor and can be recognized by the mitochondrial import machinery. On the other hand it cannot be excluded that besides the unfolding of cytosolic precursors the role of NTPs includes functions such as the modification of mitochondrial membranes e.g. by phosphorylation of mitochondrial transport components.

The existence of "unfolding proteins" in the cytosol has been proposed (Rothman and Kornberg, 1986; Zimmermann and Meyer, 1986) that could bind to precursor proteins and whose action would involve the hydrolysis of high energy phosphate bonds. The role of NTPs in preventing (mis)folding of precursor proteins into an import incompetent conformation could explain the general importance of NTPs for the translocation of proteins across membranes in both procaryotic and eucaryotic systems (for review see Zimmermann and Meyer, 1986).

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