Role of ATP in Mitochondrial Protein Import

CONFORMATIONAL ALTERATION OF A PRECURSOR PROTEIN CAN SUBSTITUTE FOR ATP REQUIREMENT*

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The role of nucleoside triphosphates (NTPs) in the import of porin into the mitochondrial outer membrane was investigated with two forms of the porin precursor: the in vitro synthesized biosynthetic precursor (bs-porin) and a water-soluble form of porin (ws-porin) obtained by subjecting the membrane-derived porin to an acid-base treatment (exposure to trichloroacetic acid, followed by alkali and rapid neutralization). The import of ws-porin into mitochondria did not require NTPs, whereas the import of bs-porin required NTPs. In other characteristics, such as binding to a specific receptor protein on the mitochondrial surface, two-step insertion into the outer membrane, and formation of specific membrane channels, ws-porin was indistinguishable from bs-porin. Thus, the acid-base treatment applied in the preparation of ws-porin can substitute for the NTP-requiring step in mitochondrial protein import. We conclude that NTPs are required for unfolding mitochondrial precursor proteins ("translocation competent folding").

The transport of cytoplasmically synthesized precursor proteins into mitochondria requires NTPs, e.g. ATP or GTP (Pfanner and Neupert, 1986; Chen and Douglas, 1987; Eilers et al., 1987; Hartl et al., 1987; Kleene et al., 1987; Pfanner et al., 1987). Several observations suggested that the NTPs are needed for the import competence of the precursor proteins rather than solely interacting with mitochondrial elements. Precursor proteins displayed different degrees of protease sensitivity at different levels of NTPs, suggesting that NTPs are affecting their folding. Chimeric precursor proteins with identical targeting signals required different concentrations of NTPs for import (Pfanner et al., 1987). Finally, import of incompletely synthesized precursor chains showed a decreased requirement for NTPs (Verner and Schatz, 1987). It has been proposed that NTPs were required to keep precursor proteins in an import-competent ("unfolded") conformation (Pfanner and Neupert, 1986; Rothman and Kornberg, 1986; Chen and Douglas, 1987; Pfanner et al., 1987; Verner and Schatz, 1987). A demonstration that unfolding a mitochondrial precursor protein, whose import requires NTPs, can bypass the requirement for NTPs would constitute direct proof for this hypothesis.

Recently, we showed that the mitochondrial import of the biosynthetic precursor form of the outer membrane protein porin (bs-porin) required NTPs (Kleene et al., 1987). A different form of porin (ws-porin) was obtained by treatment of the purified membrane protein with 5% trichloroacetic acid, followed by treatment with 100 mM NaOH and by rapid neutralization with NaH2PO4 (Pfaller et al., 1985). This ws-porin will also specifically bind to a receptor protein on the mitochondrial surface, compete with bs-porin for specific binding, insert into the outer membrane in a two-step reaction, and form specific membrane channels (Pfaller et al., 1986; Pfaller and Neupert, 1987). We now report that the import of ws-porin does not require NTPs.

EXPERIMENTAL PROCEDURES

Published procedures were used for the import of porin into isolated Neurospora crassa mitochondria (Pfaller et al., 1985; Kleene et al., 1987; Pfanner and Neupert, 1987). Either bs-porin was employed which was synthesized in rabbit reticulocyte lysates and labeled with [35S]methionine, or ws-porin was employed which was prepared from the membrane form and labeled with 13C by reductive methylation. The import reactions included mitochondria (200 μg of protein), 10-30 μl of a 100 mM phosphate buffer (pH 7), 25 μl of rabbit reticulocyte lysate, antimycin A (5 μM final concentration), oligomycin (20 μM final concentration), and a buffer consisting of 250 mM sucrose, 80 mM KCl, 5 mM MgCl2, 10 mM MOPS, and 3% (w/v) bovine serum albumin, adjusted to pH 7.2 with KOH (Pfanner and Neupert, 1985). The final volume was 200 μl.

RESULTS AND DISCUSSION

An in vitro import reaction containing Neurospora mitochondrial and rabbit reticulocyte lysate was depleted of endogenous ATP (and ADP) by preincubation with apyrase (an ATPase and an ADPase from potato) (Fig. 1, lanes 1 and 3). Control samples received an apyrase preparation heated to 95 °C prior to use (Fig. 1, lanes 2 and 4). Samples 1 and 2 contained 35S-labeled ws-porin, whereas samples 3 and 4 contained 13C-labeled bs-porin synthesized in rabbit reticulocyte lysates. Oligomycin was included to prevent further import of ATP by the FoF1-ATPase. After incubation for 15 min at 25 °C, the mitochondria were treated with proteinase K at a high concentration which completely degraded precursors which were not imported (Kleene et al., 1987; Pfaller and Neupert, 1987). Mitochondria were then resiolated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fluorographs of the dried gels are shown in Fig. 1. The import of bs-porin was inhibited by the pretreatment with apyrase (lane 3) as expected. Readdition of ATP or of GTP was shown to restore import demonstrating the NTP requirement of import of bs-porin into mitochondria (Kleene et al., 1987). The import of ws-porin, however, was not affected by the pretreatment with apyrase (lane 1) suggesting that
FIG. 1. Depletion of the \textit{in vitro} import system from ATP does not inhibit the import of \textit{ws}-porin into mitochondria. Isolated mitochondria (10 mg/ml) and reticulocyte lysate were preincubated with apyrase (8 units/ml; lanes 1 and 3) or an apyrase preparation heated to 95 °C prior to use (corresponding to 8 units/ml; lanes 2 and 4) as described (Pfanner and Neupert, 1986). The import reactions were performed as described under “Experimental Procedures.” Samples 1 and 2 contained \textsuperscript{14}C-labeled \textit{ws}-porin, and samples 3 and 4 contained \textsuperscript{35}S-labeled \textit{bs}-porin. After incubation for 15 min at 25 °C, the samples were treated with proteinase K (250 \(\mu\)g/ml) as described (Kleene et al., 1987). Mitochondria were resolated and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fluorographs of the dried gels are shown. The protein band corresponding to imported porin is marked by an \textit{arrowhead}.

FIG. 2. Import of ADP/ATP carrier and of \textit{bs}-porin, but not of \textit{ws}-porin, is inhibited by pretreatment with different concentrations of apyrase. The experiment was performed as described in the legend of Fig. 1 with the following modifications. The import reactions contained ascorbate and \(N,N,N',N'-\)tetramethylphenylenediamine as described (Pfanner and Neupert, 1987b). The reactions contained \textsuperscript{14}C-labeled \textit{ws}-porin or \textsuperscript{35}S-labeled \textit{bs}-porin or \textsuperscript{35}S-labeled precursor of the ADP/ATP carrier (AAC) together with unlabeled \textit{ws}-porin. The concentrations of apyrase were as indicated. Results were quantified by densitometry.

FIG. 3. The kinetics of import of \textit{ws}-porin are not affected by pretreatment with apyrase. The experiment was performed as described in the legends of Figs. 1 and 2. The concentration of apyrase was 80 units/ml. Time points and temperatures of import reactions were as indicated.

FIG. 4. Import of a converted form of \textit{bs}-porin is not inhibited by pretreatment with apyrase. The experiment was performed as described in the legend of Fig. 2 with the following modifications. Apyrase and \(N,N,N',N'-\)tetramethylphenylenediamine were omitted. The samples contained \textit{bs}-porin which was precipitated out of \textsuperscript{35}S-labeled reticulocyte lysate with (NH₄)₂SO₄ (64% saturation) and then treated with trichloroacetic acid, NaOH, and NaH₂PO₄, as described (Pfanner et al., 1985). NTPs were not required. The same result was obtained when the import of \textit{ws}-porin was performed in the absence of reticulocyte lysate.

In order to be sure that the samples containing \textit{ws}-porin were depleted of ATP, we performed the following experiment (Fig. 2). The \textit{in vitro} import system was pretreated with different concentrations of apyrase. The import of \textit{bs}-porin was strongly inhibited by pretreatment with a low concentration of apyrase (0.8 unit/ml), whereas the import of \textit{ws}-porin was not affected even by a 100-fold higher concentration of apyrase (80 units/ml). Import of ADP/ATP carrier was performed in the presence of unlabeled \textit{ws}-porin, and also import of this precursor was strongly inhibited by the pretreatment with apyrase. The total amount of precursor proteins in these import reactions and the protease resistance of imported porin and ADP/ATP carrier were not affected by the apyrase treatment; furthermore, the ratios between free and imported precursor proteins were similar with \textit{ws}-porin, \textit{bs}-porin, and ADP/ATP carrier under the experimental conditions applied (Pfanner and Neupert, 1986; Kleene et al., 1987; Pfanner et al., 1987; and data not shown). This suggests that \textit{ws}-porin can be imported into mitochondria in an ATP-depleted \textit{in vitro} system in contrast to \textit{bs}-porin, ADP/ATP carrier, and many other mitochondrial precursor proteins (see the Introduction).

The data in Fig. 2 did not exclude that ATP depletion might lead to a reduced rate of porin import. Therefore, we examined the kinetics of import at different temperatures (25 and 0 °C) (Fig. 3). Pretreatment with apyrase (80 units/ml) did not affect the rates of import of \textit{ws}-porin.

As a further control, the biosynthetic porin precursor was subjected to a procedure similar to that used for the preparation of \textit{ws}-porin including precipitation with trichloroacetic acid, solubilization in 100 mM NaOH, and neutralization with NaH₂PO₄. This converted \textit{bs}-porin was tested for import into mitochondria. It was efficiently inserted into the outer membrane. Pretreatment of the \textit{in vitro} import reaction with different concentrations of apyrase did not reduce import (Fig. 4). Thus, also the biosynthetic porin precursor could be converted to a form whose import was independent of NTPs.

In conclusion, two different forms of a mitochondrial precursor protein show completely different requirements for NTPs for the import into mitochondria. There are essentially two possible explanations for the nature of the NTP-requiring
step which can be bypassed by the acid-base treated precursor. NTPs might be required for release of precursor proteins from cytosolic transport complexes in which the precursors might interact with so far uncharacterized components (for review see Pfanner and Neupert, 1987a). The import of a largely purified precursor protein, however, requires NTPs (Pfanner et al., 1987). It is thus unlikely that the dissociation of complexes between precursor proteins and other components represents the crucial function of NTPs in protein import. It is much more likely that NTPs are involved in modifying the precursor proteins in such a manner that they assume a less rigid ("unfolded") conformation. In support of such a function, NTPs increase the protease sensitivity of precursor proteins in reticulocyte lysate (Pfanner et al., 1987). The proposed unfolding reaction may be mimicked by the acid-base treatment which is known to greatly destabilize the conformation of proteins. This conclusion extends recent studies which suggested that mitochondrial precursor proteins must be at least partially unfolded to be competent for transport into mitochondria (Schleyer and Neupert, 1985; Eilers and Schatz, 1986). An NTP-dependent unfolding enzyme was proposed to participate in this process (Rothman and Kornberg, 1986; Pfanner et al., 1987). It is not known if the proposed unfolding enzyme is located in the cytoplasm or associated with the mitochondrial membranes, or both. In addition, the translocation of precursors into or across the inner membrane may require NTPs for further steps, e.g. for the phosphorylation of components of the translocation machinery.

A similar role of NTPs as in unfolding precursor proteins upon import into mitochondria may exist in the case of translocation of proteins across other biological membranes and organelles such as the endoplasmic reticulum, chloroplasts, and the prokaryotic plasma membrane. An ATP requirement was also described for protein transport into or across these membranes (Grossman et al., 1980; Chen and Tai, 1985; Flügge and Hinz, 1986; Geller et al., 1986; Hansen et al., 1986; Mueckler and Lodish, 1986; Perara et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Pain and Blobel, 1987; Schlenstedt and Zimmermann, 1987; Yamane et al., 1987). Protein export in Escherichia coli was shown to require at least partial unfolding of the precursor protein (Randall and Hardy, 1986). Indeed, the ATP requirement for protein transport into the endoplasmic reticulum appears to be related to preservation of transport competence of the precursor protein (Wiech et al., 1987).

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Note Added in Proof—The converted bs-porin shows a 3-4-fold higher sensitivity toward digestion by low concentrations of proteinase K than the authentic bs-porin. This supports the conclusion that the converted precursor exists in a more loosely folded ("unfolded") conformation than the authentic one.

REFERENCES