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Role of ATP in Mitochondrial Protein Import

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

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Nikolaus Pfanner, Rupert Pfaller, Ralf Kleene, Masaki Ito‡, Maximilian Tropschug, and Walter Neupert§

From the Institut für Physiologische Chemie, Universität München, Goethestrasse 33, 8000 München 2, Federal Republic of Germany

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,[1] *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 NaH_2PO_4 (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.*, 1985; 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; Pfaller and Neupert, 1987). Either bs-porin was employed which was synthesized in rabbit reticulocyte lysates and labeled with [^{35}S] methionine, or ws-porin was employed which was prepared from the membrane form and labeled with ^{14}C 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 (8 μM final concentration), oligomycin (20 μM final concentration), and a buffer consisting of 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 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* mitochondria 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 ^{14}C-labeled ws-porin, whereas samples 3 and 4 contained ^{35}S-labeled bs-porin synthesized in rabbit reticulocyte lysates. Oligomycin was included to prevent formation of ATP by the F_oF_1-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 reisolated 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

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‡ Present address: Institute of Comprehensive Medical Science, Fujita Gakuen Health University, Toyoake, Aichi 470-II, Japan.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: NTPs, nucleoside triphosphates; bs-porin, biosynthetic porin precursor; ws-porin, water-soluble porin; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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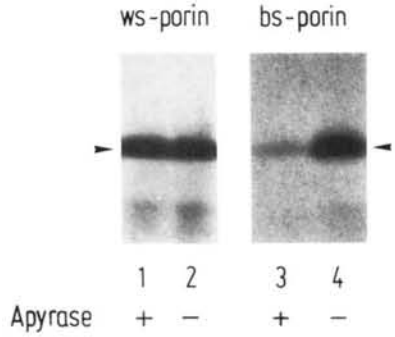

FIG. 1. **Depletion of the *in vitro* import system from ATP does not inhibit the import of ws-porin into mitochondria.** Isolated mitochondria (10 mg/ml) and reticulocyte lysate were preincubated with apyrase (8 units/ml; *lanes 1* and *3*) or with an apyrase preparation heated to 95 °C prior to use (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 ^{14}C-labeled ws-porin, and samples 3 and 4 contained ^{35}S-labeled bs-porin. After incubation for 15 min at 25 °C, the samples were treated with proteinase K (250 μg/ml) as described (Kleene *et al.*, 1987). Mitochondria were reisolated 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 *arrowhead*.

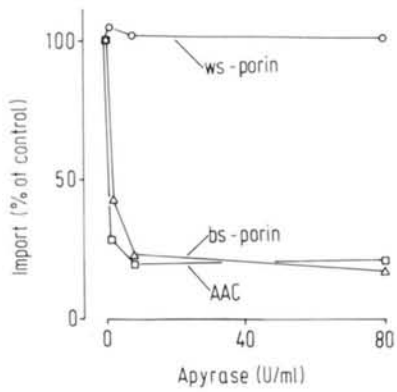

FIG. 2. **Import of ADP/ATP carrier and of bs-porin, but not of ws-porin, is inhibited by pretreatment with different concentrations of apyrase.** The experiment was performed as described in the legend of Fig. 1 with the following modifications. The import reactions contained ascorbate and *N,N,N′,N′*-tetramethylphenylenediamine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

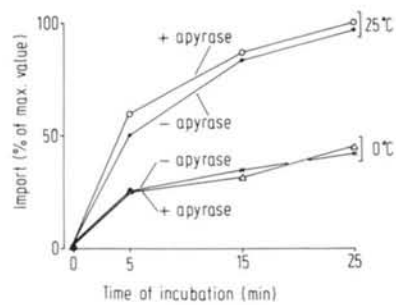

FIG. 3. **The kinetics of import of ws-porin are not affected by pretreatment with apyrase.** The experiment was performed as described in the legends of Figs. 1 and 2. The concentration of apyrase was 80 units/ml. Time points and temperatures of import reactions were as indicated.

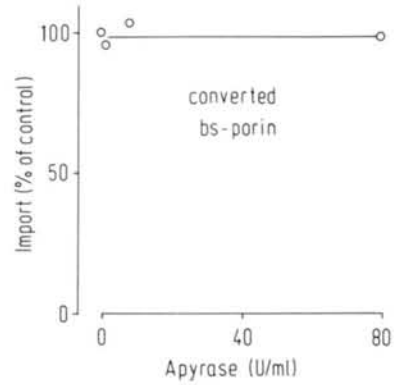

FIG. 4. **Import of a converted form of bs-porin is not inhibited by pretreatment with apyrase.** The experiment was performed as described in the legend of Fig. 2 with the following modifications. Ascorbate and *N,N,N′,N′*-tetramethylphenylenediamine were omitted. The samples contained bs-porin which was precipitated out of ^{35}S-labeled reticulocyte lysate with $(NH_4)_2SO_4$ (64% saturation) and then treated with trichloroacetic acid, NaOH, and NaH_2PO_4 as described (Pfaller *et al.*, 1985).

NTPs were not required. The same result was obtained when the import of ws-porin was performed in the absence of reticulocyte lysate.

In order to be sure that the samples containing ws-porin were depleted of ATP, we performed the following experiment (Fig. 2). The *in vitro* import system was pretreated with different concentrations of apyrase. The import of bs-porin was strongly inhibited by pretreatment with a low concentration of apyrase (0.8 unit/ml), whereas the import of 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 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 ws-porin, 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 ws-porin can be imported into mitochondria in an ATP-depleted *in vitro* system in contrast to 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 ws-porin.

As a further control, the biosynthetic porin precursor was subjected to a procedure similar to that used for the preparation of ws-porin including precipitation with trichloroacetic acid, solubilization in 100 mM NaOH, and neutralization with NaH_2PO_4. This converted bs-porin was tested for import into mitochondria. It was efficiently inserted into the outer membrane. Pretreatment of the *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

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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, also requires NTPs (Eilers *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.

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