Mitochondrial Protein Import: Nucleoside Triphosphates Are Involved in Conferring Import-Competence to Precursors

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Summary

The role of nucleoside triphosphates (NTPs) in mitochondrial protein import was investigated with the precursors of N. crassa ADP/ATP carrier, F_1-ATPase subunit β, F_0-ATPase subunit 9, and fusion proteins between subunit 9 and mouse dihydrofolate reductase. NTPs were necessary for the initial interaction of precursors with the mitochondria and for the completion of translocation of precursors from the mitochondrial surface into the mitochondria. Higher levels of NTPs were required for the latter reactions as compared with the early stages of import. Import of precursors having identical presequences but different mature protein parts required different levels of NTPs. The sensitivity of precursors in reticulocyte lysate to proteases was decreased by removal of NTPs and increased by their readdition. We suggest that the hydrolysis of NTPs is involved in modulating the folding state of precursors in the cytosol, thereby conferring import competence.

Introduction

Transport of cytoplasmically synthesized precursor proteins into mitochondria involves several consecutive steps (for review see Hay et al., 1984; Harmey and Neupert, 1985; Pfanner and Neupert, 1987a). The precursors bind to specific receptor sites on the mitochondrial surface (Hennig and Neupert, 1981; Gasser et al., 1982; Hennig et al., 1983; Zwizinski et al., 1983, 1984; Riezman et al., 1983; Pfaller et al., 1985; Schmidt et al., 1985; Hartl et al., 1986; Pfanner and Neupert, 1987b). Translocation into or across the inner mitochondrial membrane takes place at contact sites between the outer and inner membranes (Schleyer and Neupert, 1985; Hartl et al., 1986; Pfanner and Neupert, 1987b; Schwaiger et al., 1987) and requires the electrical potential ($\Delta\psi$) across the inner membrane (Schleyer et al., 1982; Gasser et al., 1982; Kolanski et al., 1982; Pfanner and Neupert, 1985). The role of $\Delta\psi$ is assumed to be an electrophoretic effect on positively charged parts of the precursor molecules (Pfanner and Neupert, 1985; Roise et al., 1986). The import of F_0F_1-ATPase subunit β (F_1β) has been demonstrated to require both $\Delta\psi$ and nucleoside triphosphates (NTPs). Non-hydrolyzable ATP analogues are not able to fulfill the NTP requirement, suggesting that hydrolysis of high energy phosphate bonds is necessary (Pfanner and Neupert, 1986). Precursor proteins with amino-terminal prese-

quences are proteolytically cleaved by the processing peptidase located in the matrix (Böhni et al., 1980, 1983; Conboy et al., 1982; McAda and Douglas, 1982; Miura et al., 1982; Zwizinski and Neupert, 1983; Schmidt et al., 1984). The presequences apparently carry sufficient information for the targeting of proteins to mitochondria (Hurt et al., 1984, 1985; Horwich et al., 1985; Emr et al., 1986; Keng et al., 1986; van Loon and Young, 1986). Presequences also seem to be important for the intramitochondrial sorting of precursors (van Loon et al., 1986; Hartl et al., 1986).

For several precursor proteins, the import pathway has been resolved into distinct steps. For example, the import of F_1β and subunits IV (cytochrome c_1) and V (FeS protein) of the bc_1-complex can be blocked at the level of translocation contact sites (Schleyer and Neupert, 1985; Hartl et al., 1986; see Figure 8A, stage b). At this stage, the amino-terminal parts of the precursors are exposed to the mitochondrial matrix, where they are proteolytically processed, whereas carboxy-terminal parts are still outside the outer membrane. These translocational intermediates can be completely imported by relieving the translocation block.

The import of ADP/ATP carrier involves the following steps (the distinct forms of ADP/ATP carrier on the import pathway are named from stage 1 to stage 5 [Pfanner and Neupert, 1987b; see Figure 8B]). The precursor is synthesized in the cytoplasm without a cleavable presequence (stage 1) (Zimmermann et al., 1979; Arends and Sebald, 1984). It binds to receptor sites on the mitochondrial surface (stage 2) and is then directed to protease-protected sites in the outer membrane (stage 3). The $\Delta\psi$-dependent entrance into the inner membrane occurs at contact sites (stage 4). The completion of translocation into the inner membrane and the assembly to the functional dimer (stage 5) do not require $\Delta\psi$.

To investigate the role of NTPs in the transport of proteins into mitochondria, the distinct steps on the import pathways of F_1β and ADP/ATP carrier were tested for their requirement of NTPs. We find that several steps need NTPs independently of whether the specific binding to mitochondria or the $\Delta\psi$-dependent step have already taken place. The common feature of the NTP-requiring steps is that they all involve translocation of parts of precursors from the cytosol into the mitochondrial membranes. Resistance to so-called unspecific proteases (e.g., proteinase K) has been used to test the degree of folding of proteins: a high protease resistance suggests a high degree of tertiary structure, whereas proteins with less tertiary structure are usually very sensitive to unspecific proteases (for review see Jaenicke and Rudolph, 1986; Randall and Hardy, 1986). In the absence of NTPs, the protease resistance of mitochondrial precursor proteins in rabbit reticulocyte lysate was increased, whereas addition of NTPs decreased the protease resistance. We conclude that the role of NTPs in mitochondrial protein import involves unfolding of precursor proteins in the cytosol.
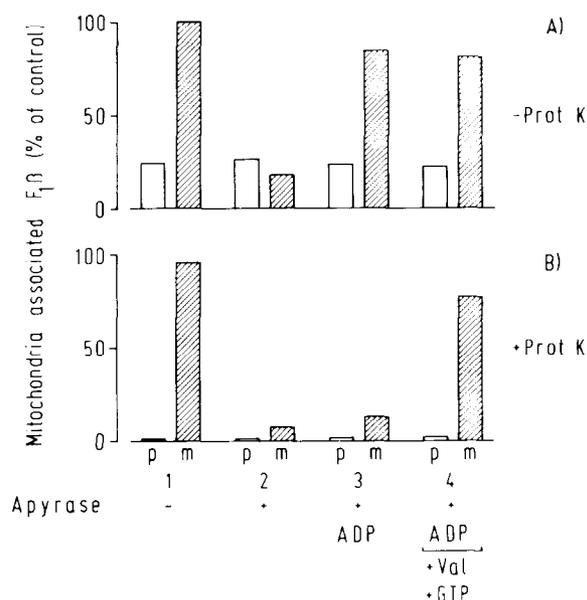
Figure 2. Import of ADP/ATP Carrier Requires NTPs

Mitochondria and reticulocyte lysate were incubated with apyrase (80 U/ml for reaction 3; 0.5 U/ml for reactions 4–6), an apyrase preparation that had been heated before use (corresponding to 80 U/ml; reaction 2), or water (reaction 1). Mitochondria and reticulocyte lysate were combined and incubated in the presence of antimycin A, oligomycin, potassium ascorbate, TMPD, and BSA-containing buffer. Reactions 5 and 6 contained 8 mM ATP or GTP. After 25 min at 25°C, samples were treated with proteinase K (200 μg/ml, final concentration). Mitochondria were reisolated, and assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography. (+), 0.5 U of apyrase per ml; (++), 80 U of apyrase per ml.

Figure 1. Transport of F₁β into Contact Sites and Completion of Import Require NTPs

Isolated mitochondria (2.5 mg of mitochondrial protein per ml) and reticulocyte lysate (containing radiolabeled precursor proteins) were treated with apyrase (0.5 U/ml) (reactions 2–4) or received an apyrase preparation that had been heated to 95°C before use (corresponding to 0.5 U/ml) (reaction 1). Mitochondria were added to the reticulocyte lysate in a mixture containing antimycin A, oligomycin, potassium ascorbate, TMPD, and BSA-containing buffer (for details see Experimental Procedures; Pfanner and Neupert, 1986). Reactions 3 and 4 contained 6 mM ADP. All samples were incubated for 25 min at 25°C. Reactions 1–3 were divided, and one-half was treated with proteinase K (15 μg/ml, final concentration) (B; + Prot K). Mitochondria of reaction 4 were reisolated and resuspended in BSA-containing buffer in the presence of valinomycin (Val); GTP was added (6 mM, final concentration), and the sample was incubated at 25°C for 15 min. The reaction was then divided, and one-half was treated with proteinase K (15 μg/ml, final concentration). Mitochondria were reisolated from all samples. F₁β was immunoprecipitated, and samples were resolved by SDS–polyacrylamide gel electrophoresis. Quantitation of the fluorograms was performed by densitometry. p, precursor of F₁β; m, mature-sized form of F₁β.

Results

Insertion of the Precursor to F₁β into Translocation Contact Sites and the Completion of Translocation into the Matrix Require NTPs

Preincubation of reticulocyte lysate, containing in vitro–synthesized mitochondrial precursor proteins, and of mitochondria with apyrase (an adenosine 5'-triphosphatase and an adenosine 5'-diphosphatase) inhibited the import of F₁β into mitochondria. Readdition of ATP or GTP restored import. A mitochondrial membrane potential was established by addition of ascorbate plus N,N,N',N'-tetramethylphenylenediamine (TMPD), which supplies electrons at the level of complex IV of the electron transport chain (Nicholls, 1982; Wikstrom and Krab, 1982). Oligomycin was added to prevent dissipation of Δψ and formation of ATP by the F₀F₁-ATPase. These studies showed that the import of F₁β needs NTPs in addition to

a membrane potential (Pfanner and Neupert, 1986). This system was also used for the experiments described in this report.

After treatment with apyrase (0.5 U/ml), reticulocyte lysate and mitochondria were mixed and incubated at 25°C; specific processing of F₁β (Figure 1A, reaction 2) and transport to a protease-protected location (Figure 1B, reaction 2) were strongly inhibited. The control (Figure 1, reaction 1) showed processing of F₁β and protection of mature-sized F₁β (m-F₁β) from proteases; protease protection is correlated with transport of F₁β into the matrix (Zwizinski and Neupert, 1983; Schleyer and Neupert, 1985). When, after treatment with apyrase, ADP was added, the specific processing to m-F₁β was restored (Figure 1A, reaction 3). This m-F₁β, however, was still sensitive to proteases from the outside of mitochondria (Figure 1B, reaction 3). Thus, the amino terminus of this F₁β had already entered the matrix space, whereas a major portion was still outside the outer membrane; apparently, this m-F₁β was spanning translocation contact sites (Schleyer and Neupert, 1985; Hartl et al., 1986). The mitochondria of reaction 4 were reisolated after incubation in the presence of ADP. They were resuspended in buffer containing bovine serum albumin (BSA) (Pfanner and Neupert, 1985) in the absence of a membrane potential (Schleyer and Neupert, 1985; Hartl et al., 1986; Pfanner and Neupert, 1987b). GTP was added, and a second incubation at 25°C was performed. Thereby, the m-F₁β that was spanning contact sites was chased to protease-protected m-F₁β (Figure 1B. reaction 4).

The restoration of processing by the addition of ADP, but not of ATP, could be inhibited by P¹,P⁵-bis-(adenosine-5') pentaphosphate (Ap₅A), an inhibitor of adenylate kinase that catalyzes the reaction 2 ADP ⇌ ATP + AMP (for review see Frank et al., 1984) (data not shown). The restoration is therefore dependent on ATP generated from ADP (addition of AMP did not lead to restoration). The ac-
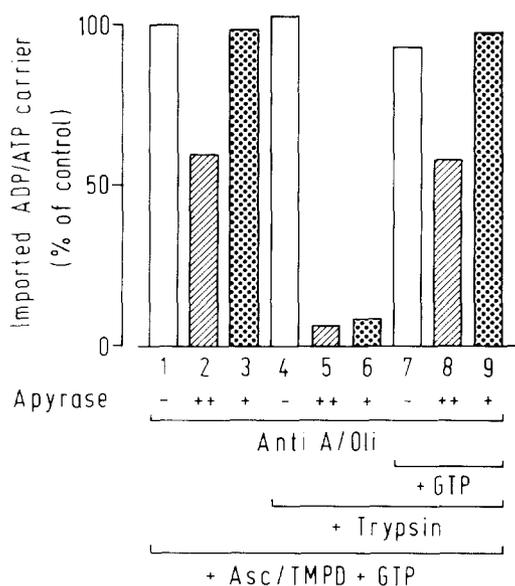
Figure 3. Binding of ADP/ATP Carrier to the Mitochondrial Surface and Insertion into the Outer Membrane Require NTPs

Mitochondria and reticulocyte lysate were incubated with an apyrase preparation that had been heated before use (corresponding to 80 U/ml, mixture a), apyrase (80 U/ml, mixture b), or apyrase (0.5 U/ml, mixture c). Mitochondria and reticulocyte lysate were combined and incubated in the presence of antimycin A (Anti A), oligomycin (Oli), and BSA-containing buffer for 25 min at 25°C. Mitochondria were reisolated and resuspended in BSA-containing buffer in the presence of antimycin A, oligomycin, and an apyrase preparation that had been heated before use (corresponding to 0.2 U/ml, mixture a) or apyrase (0.2 U/ml, mixtures b and c). Each mixture was divided into three portions (mixture a into reactions 1, 4, and 7; mixture b into 2, 5, and 8; mixture c into 3, 6, and 9). One set of reactions (4–6) was treated with trypsin (15 μg/ml final concentration). Reactions 1–3 received the same volume of SEM medium. Reactions 7–9 received 6 mM GTP and were incubated for 15 min at 25°C, followed by treatment with trypsin (15 μg/ml, final concentration). All reactions received potassium ascorbate (Asc), TMPD, and 8 mM GTP and were incubated for 15 min at 25°C. Mitochondria were reisolated again, and assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography. (+), 0.5 U of apyrase per ml; (++), 80 U of apyrase per ml.

cumulation of m-F₁β that was spanning translocation contact sites seen in reaction 3 of Figure 1 was also obtained when the pretreatment was performed with lower concentrations of apyrase (without addition of ADP) or when at higher apyrase concentrations, ATP was added.

In summary, transport of $F_1\beta$ into contact sites requires NTPs. The completion of import, however, needs higher levels of NTPs (see Figure 8A). The completion is independent of the membrane potential (Schleyer and Neupert, 1985).

Specific Binding of ADP/ATP Carrier to the Mitochondrial Surface and Insertion into the Outer Membrane Require NTPs

Reticulocyte lysate and mitochondria were pretreated with apyrase as described for the experiments with $F_1\beta$ (see above; Pfanner and Neupert, 1986). Under these conditions, the import of ADP/ATP carrier was also inhibited

(Figure 2, reactions 3 and 4). Addition of ATP or GTP restored import (Figure 2, reactions 5 and 6); the import was even stimulated over the control values (reactions 1 and 2). The import of ADP/ATP carrier into the inner membrane was tested by protection against high concentrations of proteinase K and by the carboxyatractyloside-dependent passage over hydroxylapatite, a convenient assay for the assembly of the ADP/ATP carrier (Schleyer and Neupert, 1984; Pfanner and Neupert, 1987b).

Which steps on the import pathway of ADP/ATP carrier need NTPs? In the absence of Δψ, the import of ADP/ATP carrier into the inner membrane is blocked, but the precursor to the ADP/ATP carrier can still bind to specific sites on the mitochondrial surface from which it can be imported after reestablishing Δψ (see Introduction; Zwizinski et al., 1983; Pfanner and Neupert, 1987b). In the experiment described in Figure 3, reticulocyte lysate and mitochondria were pretreated with apyrase (80 U/ml or 0.5 U/ml). Controls received an apyrase preparation that had been heated before use. Reticulocyte lysate and mitochondria were then mixed and incubated at 25°C in the presence of antimycin A and oligomycin, which destroy the mitochondrial membrane potential (Nicholls, 1982; Wikstrom and Krab, 1982). Mitochondria were reisolated and resuspended in BSA-containing buffer in the presence of antimycin A, oligomycin, and apyrase. The mixtures were divided into three portions, each. The first portions (Figure 3, reactions 1–3) were left untreated, whereas the second portions (reactions 4–6) were treated with trypsin at a concentration that preserves the stage 3 intermediate in the outer membrane but degrades the receptor-bound stage 2 intermediate (see Introduction; Pfanner and Neupert, 1987b). A membrane potential was then established in both groups by addition of ascorbate plus TMPD, and NTPs were added. After incubation at 25°C, mitochondria were reisolated again and assayed for imported ADP/ATP carrier. Specific (i.e., import-competent) binding of ADP/ATP carrier still occurred after treatment with apyrase (Figure 3, reactions 2 and 3). This specifically bound precursor was sensitive to trypsin treatment (Figure 3, reactions 5 and 6) (stage 2 intermediate), whereas in the control reaction (reaction 4) the precursor was already inserted into a protease-protected location in the outer membrane (stage 3). The third group of portions (Figure 3, reactions 7–9) received NTPs, in the absence of Δψ, after the first reisolation of mitochondria and was then incubated at 25°C. Treatment with trypsin and further additions were performed as described for reactions 4–6. Thereby, the stage 2 intermediate could be chased to the protease-protected stage 3 intermediate, and then to assembled inner membrane ADP/ATP carrier (Figure 3, reactions 8 and 9).

We conclude that the insertion of the ADP/ATP carrier into the outer membrane strongly depends on the presence of NTPs. Since pretreatment with high apyrase concentrations reduced the amount of specifically bound precursor (Figure 3, reactions 2 and 8), binding to receptor sites on the mitochondrial surface also seems to require NTPs.
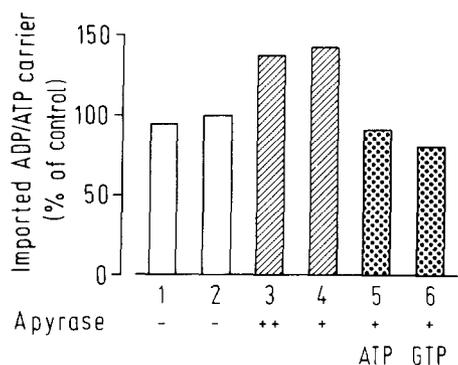
Figure 4. Transport of ADP/ATP Carrier from the Outer Membrane into the Inner Membrane Is Stimulated by Removal of NTPs

Mitochondria, reticulocyte lysate, antimycin A, and oligomycin were incubated for 25 min at 25°C. Mitochondria were reisolated and suspended in BSA-containing buffer in the presence of antimycin A and oligomycin. Samples were incubated with apyrase (20 U/ml for reaction 3; 0.13 U/ml for reactions 4–6), an apyrase preparation that had been heated before use (corresponding to 20 U/ml for reaction 2), or water (reaction 1) for 20 min at 25°C. All samples received potassium ascorbate and TMPD. In addition, sample 5 received 8 mM ATP and sample 6 received 8 mM GTP. All samples were incubated for 15 min at 25°C and then treated with proteinase K (200 μg/ml, final concentration). Mitochondria were reisolated, and assembled ADP/ATP carrier was assessed by hydroxylapatite chromatography. Similar results were obtained when the import of ADP/ATP carrier was determined by protection against high concentrations of proteinase K (200 μg/ml). (+) 0.13 U of apyrase per ml; (++), 20 U of apyrase per ml.

Transport of ADP/ATP Carrier from the Outer Membrane into the Inner Membrane and Assembly to the Dimeric Form Do Not Require NTPs

The precursor to the ADP/ATP carrier was bound to mitochondria in the absence of $\Delta\psi$ at 25°C. Thereby, the stage 3 intermediate, which is inserted into the outer membrane, was generated. After reisolation, mitochondria were resuspended in BSA-containing buffer in the absence of a $\Delta\psi$ and treated with apyrase (Figure 4, reactions 3–6) or not treated (reactions 1 and 2). A membrane potential was then established. In addition, reactions 5 and 6 (Figure 4) received NTPs. After incubation at 25°C, imported and assembled ADP/ATP carrier was determined. The removal of NTPs did not inhibit the import of the stage 3 ADP/ATP carrier, but rather stimulated it (Figure 4, reactions 3 and 4). Readdition of NTPs (Figure 4, reactions 5 and 6) reduced the translocation of precursors from the outer into the inner membrane to (and even below) the control values (reactions 1 and 2). In separate experiments it was demonstrated for endogenous mitochondrial ADP/ATP carrier that the protease resistance in intact mitochondria and the ability to pass over hydroxylapatite were not affected by modifying the concentrations of NTPs (data not shown).

In summary, the various steps on the import pathway of ADP/ATP carrier require different levels of NTPs. Binding to receptor sites on the mitochondrial surface requires lower levels than insertion into the outer membrane. There is no demonstrable requirement for transport from the outer into the inner membrane (see Figure 8B).
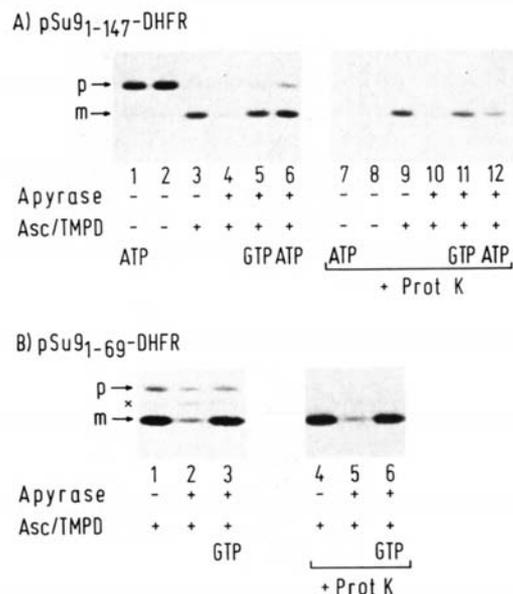

Figure 5. Import of ATPase Subunit 9–DHFR Fusion Proteins Requires NTPs

(A) Mitochondria and reticulocyte lysate (containing pSu9$_{1-147}$-DHFR) were incubated with apyrase (0.5 U/ml, reactions 4–6 and 10–12) or an apyrase preparation that had been heated before use (corresponding to 0.5 U/ml, reactions 1–3 and 7–9). Mitochondria and reticulocyte lysate were combined and incubated in the presence of antimycin A, oligomycin, and BSA-containing buffer. Reactions 3–6 and 9–12 contained potassium ascorbate (Asc) and TMPD. Reactions 1, 6, 7, and 12 contained 8 mM ATP. Reactions 5 and 11 contained 8 mM GTP. All reactions were incubated for 25 min at 25°C. Reactions 7–12 were treated with proteinase K (25 μg/ml final concentration) (Prot K). Mitochondria were reisolated, and samples were resolved by SDS–polyacrylamide gel electrophoresis. p, precursor form; m, mature-sized form.
(B) The experiment was performed as described for reactions 3–5 and 9–11 of (A) with the following modifications. Reticulocyte lysate containing pSu9$_{1-69}$-DHFR was used. The apyrase concentration was 16 U/ml. p, precursor form; m, mature-sized form. The band indicated with "x" could represent a processing intermediate of the fusion protein. Such a processing intermediate, with the same differences in apparent molecular weight between precursor form and mature form, has been identified for native ATPase subunit 9 by radiosequencing (Schmidt et al., 1984).

Fusion Proteins Containing Nonmitochondrial Polypeptides Also Require NTPs for Import into Mitochondria

Is the requirement for NTPs a general phenomenon of mitochondrial protein import? We studied whether proteins containing large nonmitochondrial parts also show this requirement. The following fusion proteins were constructed by combining different parts of the coding region for the precursor to F$_0$-ATPase subunit 9 of Neurospora crassa (Viebrock et al., 1982) and for mouse dihydrofolate reductase (DHFR) (Nunberg et al., 1980), a cytosolic protein: complete subunit 9 precursor (amino-terminal) + DHFR (carboxy-terminal) (pSu9$_{1-147}$-DHFR; Figure 5A); presequence and 3 amino acids of the mature subunit 9 (amino-terminal) + DHFR (carboxy-terminal) (pSu9$_{1-69}$-DHFR; Figure 5B).

Both fusion proteins were processed by isolated Neurospora mitochondria (Figure 5A, reaction 3; Figure 5B,
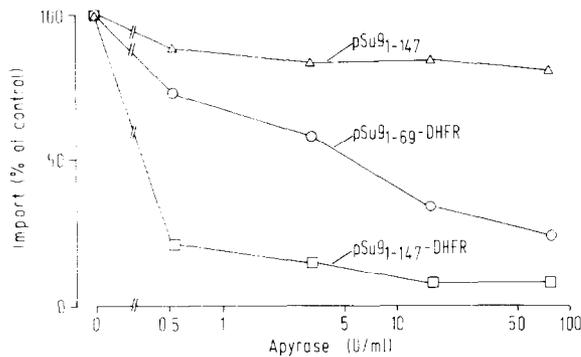
Figure 6. Import of Precursors Having Identical Presequences but Different Mature Protein Parts Requires Different Levels of NTPs

The experiment was performed as described in Figure 5A, reactions 3 and 4, with the following modifications. Reticulocyte lysate contained the precursors to either native ATPase subunit 9 (pSu9$_{1-147}$) or fusion proteins pSu9$_{1-69}$-DHFR or pSu9$_{1-147}$-DHFR. The apyrase concentrations used were as indicated. Results were quantified by densitometry of the bands corresponding to the mature-sized proteins. Similar results were obtained when protease protection of the mature-sized fusion proteins was tested.

A)

B)

Figure 7. NTPs Decrease the Protease Resistance of In Vitro- Synthesized Precursor Proteins

(A) Reticulocyte lysate (12 μl, containing pSu9$_{1-147}$-DHFR) was incubated with apyrase (1.3 U/ml, reactions 3–5) or an apyrase preparation that had been heated before use (corresponding to 1.3 U/ml, reactions 1 and 2) for 20 min at 30°C and 20 min at 25°C and cooled to 0°C. BSA-containing buffer (12 μl) was then added. Reaction 4 received 5 mM ATP. Reaction 5 received 5 mM GTP. All reactions were incubated for 10 min at 25°C and cooled to 0°C. Reactions 4 and 5 again received the same amounts of ATP and GTP as described above. Reactions 2–5 were then treated with proteinase K (Prot K) (1 μg/ml, final concentration). Samples were resolved by SDS–polyacrylamide gel electrophoresis. Undigested pSu9$_{1-147}$-DHFR fusion protein was quantified by densitometry.

(B) The experiment was performed as described for (A), with the following modifications. Reticulocyte lysate (50 μl, containing precursor to ADP/ATP carrier synthesized out of poly(A)$^+$ RNA) was incubated with apyrase (30 U/ml, reactions 2, 3, 5, 6, 8, and 9) or an apyrase preparation that had been heated before use (corresponding to 30 U/ml, reactions 1, 4, and 7); 220 μl of BSA-containing buffer was added. Reactions 3, 6, and 9 received 8 mM GTP. The concentrations of proteinase K were as indicated. ADP/ATP carrier was immunoprecipitated and quantified by densitometry of the fluorograms.

reaction 1). Incubation of the fusion proteins with the purified processing peptidase of the mitochondrial matrix yielded products with the same apparent molecular weight (Hawlitschek and Neupert, unpublished data). The mature-sized proteins generated by intact mitochondria were protected against externally added proteases (Figure 5A, reaction 9; Figure 5B, reaction 4). Dissipation of Δψ, by addition of antimycin A plus oligomycin, inhibited the processing and transport to a protease-protected location (Figure 5A, reactions 2 and 8; and data not shown). ATP could not substitute for the requirement of Δψ (Figure 5A, reactions 1 and 7). When mitochondria and reticulocyte lysate were pretreated with apyrase, then mixed together and a membrane potential established, the processing and transport to a protease-protected location were inhibited (Figure 5A, reactions 4 and 10; Figure 5B, reactions 2 and 5). Readdition of NTPs restored the import of the fusion proteins (Figure 5A, reactions 5, 6, 11, and 12; Figure 5B, reactions 3 and 6). Thus, the import of the fusion proteins needs both Δψ and NTPs.

Import of Precursors Having Identical Presequences but Different Mature Protein Parts Requires Different Levels of NTPs

Mitochondria and reticulocyte lysate were pretreated with different concentrations of apyrase. Then, the proteolytic processing and transport to a protease-protected location were tested for both fusion proteins and the native subunit 9 precursor (Figure 6). The import of subunit 9 precursor (pSu9$_{1-147}$) was only slightly inhibited, even with high apyrase concentrations. The import of the longer fusion protein (pSu9$_{1-147}$-DHFR) was already inhibited at low apyrase concentrations. The import of the shorter fusion protein (pSu9$_{1-69}$-DHFR) was slightly inhibited with low apyrase concentrations, but more strongly inhibited with higher apyrase concentrations.

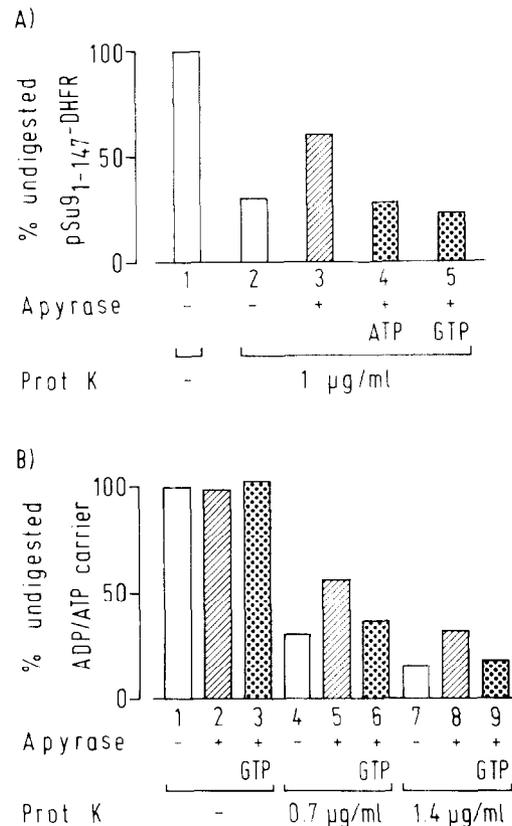Thus, the import of three precursors having identical presequences but different mature protein parts shows different sensitivity toward removal of NTPs.

NTPs Decrease the Protease Resistance of Precursor Proteins in Rabbit Reticulocyte Lysate

Resistance against proteinase K has been used as a criterion for the folding state of a protein (see Introduction). The fusion protein pSu9$_{1-147}$-DHFR was synthesized in rabbit reticulocyte lysate (Figure 7A, reaction 1) and treated with proteinase K at a concentration that degraded

A) ATPase $F_1\beta$

NTP dependent
unfolding

B) ADP/ATP carrier
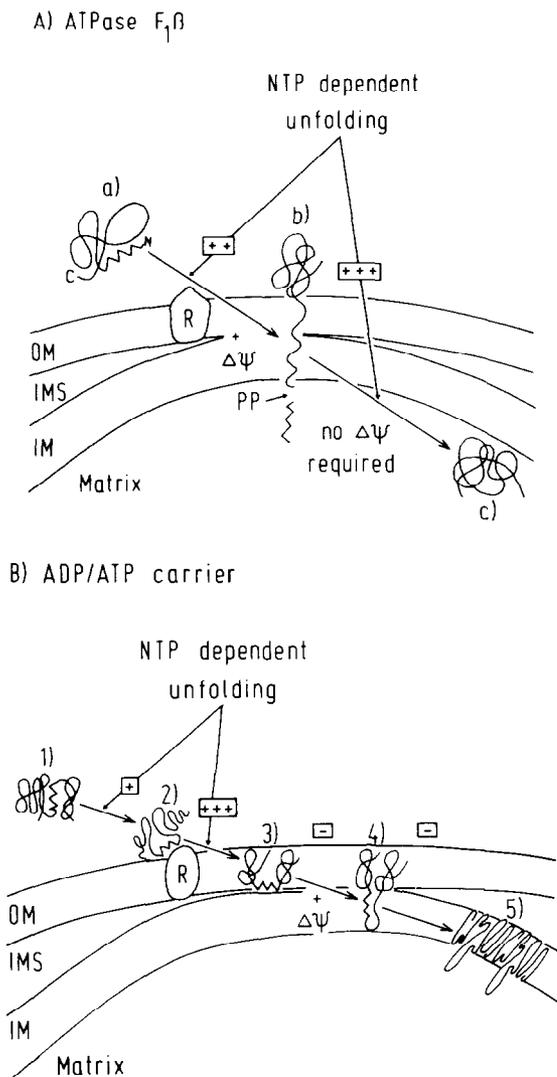
NTP dependent
unfolding

Figure 8. Distinct Steps on the Import Pathway of $F_1\beta$ and ADP/ATP Carrier into Mitochondria Require NTPs

The requirements for NTPs are indicated in squares from high requirement (+++) to no requirement (-). The various translocational intermediates are as described in the text and as published previously (Schleyer and Neupert, 1985; Pfanner and Neupert, 1987b). The topology of the mature ADP/ATP carrier in the inner membrane was drawn as proposed by Bogner et al. (1982, 1986). OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner membrane; N, amino terminus; C, carboxyl terminus; R, receptor; PP, processing peptidase; $\Delta\psi$, membrane potential.

70% of the precursors (Figure 7A, reaction 2). Pretreatment of the reticulocyte lysate (containing the precursor) with apyrase increased the protease resistance of the precursors (Figure 7A, reaction 3). Readdition of ATP or GTP decreased the protease resistance to the control value (Figure 7A, reactions 4 and 5). Similarly, pretreatment with apyrase increased the protease resistance of the precursor to the ADP/ATP carrier (Figure 7B, reactions 5 and 8); the protease concentrations were such that 70% (reaction 4) and 85% (reaction 7) of the precursors were degraded under control conditions. Readdition of NTPs decreased the protease resistance (Figure 7B, reactions

6 and 9). Thus, the presence of NTPs seems to influence the folding state of mitochondrial precursor proteins, i.e., promote unfolding.

Discussion

The import of several mitochondrial precursor proteins was shown to need both $\Delta\psi$ and NTPs. Distinct steps on the import pathways of $F_1\beta$ (Schleyer and Neupert, 1985) and ADP/ATP carrier (Pfanner and Neupert, 1987b) were investigated for the requirement of NTPs. The results are summarized in Figure 8.

Transport of the cytosolic precursor to $F_1\beta$ (Figure 8A, stage a) to a translocation intermediate that reaches from the outside of the outer membrane into the matrix, such that the presequence can be cleaved off by the processing peptidase (stage b), requires NTPs. Further transport of the large carboxy-terminal portion from the cytosol into the matrix (stage c) requires higher levels of NTPs in a step that can occur in the absence of $\Delta\psi$.

Binding of the cytosolic precursor to the ADP/ATP carrier (Figure 8B, stage 1) to receptor sites on the mitochondrial surface (stage 2) shows a (slight) dependence on NTPs. Insertion of the precursor into the outer membrane (stage 3) requires higher levels of NTPs. These steps can take place in the absence of $\Delta\psi$. The $\Delta\psi$-dependent entrance of the precursor into the inner membrane via translocation contact sites (stage 4), the completion of translocation into the inner membrane, and the assembly to the dimeric form (stage 5), however, are stimulated by removal of NTPs. Since the precursor to the ADP/ATP carrier is synthesized without a cleavable presequence, it is clear that the proteolytic processing of precursors is not the primary reason for the need of NTPs. A requirement for NTPs has also been shown for the import of porin into the outer membrane; this import occurs without proteolytic cleavage and is independent of $\Delta\psi$ (Kleene, Pfanner, Pfaller, Sebald, Neupert, and Tropschug, submitted).

The import of three precursors, each containing the presequence of F_0-ATPase subunit 9 but different mature protein parts, was investigated for requirement of NTPs. Despite that they supposedly use the same import sites, their imports show different requirements for NTPs.

In summary, several steps in the import of proteins into mitochondria require NTPs. The common feature of these steps appears to be the translocation of precursor domains from the cytosol onto or into the mitochondrial outer membrane. The complete insertion of precursors into the outer membrane needs higher levels of NTPs than either partial insertion or binding to receptor sites on the mitochondrial surface. The results are not consistent with models that explain the requirement for NTPs only by modification of the mitochondria, e.g., by phosphorylation of receptors or of other components of the mitochondrial import machinery, or by providing energy for the action of mitochondrial transport proteins. We have suggested as a possible explanation for this pleiotropic effect that the cytosolic conformation of mitochondrial precursor proteins is modified by the aid of NTPs (Pfanner and Neupert, 1986). Eilers and Schatz (1986) have shown that a high degree of tertiary structure hinders the import of a protein

into mitochondria. Using resistance against digestion by proteinase K as a criterion for the folding state of mitochondrial precursor proteins, we could indeed show that the presence of NTPs leads to an unfolding of the precursors. Thus, the role of NTPs includes cytosolic unfolding of precursors or precursor parts to make them competent for translocation into the mitochondrial membranes. Modifications of the mitochondrial membranes in addition to cytosolic conformational changes, however, cannot be excluded.

How do NTPs modulate the conformation of precursor proteins? The data reported here are consistent with proposed cytosolic activities able to unfold proteins (Rothman and Kornberg, 1986; Zimmermann and Meyer, 1986). We suggest the following model: cytosolic protein(s) binds to mitochondrial precursor proteins and keeps them in import-competent conformation, thereby preventing the (mis)folding of precursors, which would otherwise make them import-incompetent. The precursors can then bind to receptor sites and be inserted into the outer membrane. In these processes, the hydrolysis of high energy phosphate bonds of NTPs is necessary. Since the insertion into the membrane requires folding of large precursor parts into competent conformation, this step needs higher amounts of NTPs than the binding to receptors. For the completion of translocation of precursors from the outer into or across the inner membrane, however, the cytosolic protein(s) must be removed. Depletion of NTPs from mitochondria may facilitate the removal, thereby stimulating the completion of import. Additional studies have to clarify the nature and exact function of the putative cytosolic cofactor(s) and determine whether they are related to cofactors described earlier (Argan et al., 1983; Miura et al., 1983; Firgaira et al., 1984; Ohta and Schatz, 1984; Argan and Shore, 1985; Pfanner and Neupert, 1987b). It is not yet known which form of NTPs, e.g., ATP or GTP, is the direct energy source.

The import of physiological mitochondrial precursor proteins is not the only example in which NTPs are needed. Fusion proteins containing nonmitochondrial polypeptides also show this requirement, suggesting a general mechanism for the unfolding of proteins. Furthermore, NTP-dependent unfolding proteins could also be necessary for the translocation of proteins across other biological membranes. Requirements for NTPs have been reported for protein export in E. coli (Chen and Tai, 1985; Geller et al., 1986), protein import into chloroplasts (Grossman et al., 1980; Flügge and Hinz, 1986), and protein translocation across the membrane of the endoplasmic reticulum (Hansen et al., 1986; Mueckler and Lodish, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Schlenstedt and Zimmermann, 1987; Wiech et al., 1987). Transport of proteins from the mitochondrial matrix into or across the inner membrane, which is analogous to protein export in E. coli (Hartl et al., 1986; Pfanner and Neupert, 1987a), might also require NTPs.

Experimental Procedures

In Vitro Import of Precursors into Mitochondria
The following procedures were performed as published previously: growth of N. crassa (wild type 74A) (Schleyer et al., 1982); isolation of

mitochondria by Percoll density gradient centrifugation (Hartl et al., 1986; Pfanner and Neupert, 1986), except that the mitochondria were washed twice in SEM medium (250 mM sucrose, 1 mM EDTA, 10 mM 3-(N-morpholino)propanesulfonic acid [MOPS], adjusted to pH 7.2 with KOH); protein determination (Bradford, 1976); synthesis of mitochondrial precursor proteins in rabbit reticulocyte lysates (Pelham and Jackson, 1976; Schleyer et al., 1982; Pfanner and Neupert, 1985); preparation of "no synthesis lysates" lacking mitochondrial precursor proteins (Pfanner and Neupert, 1985); treatment of reticulocyte lysate and mitochondria with apyrase (Pfanner and Neupert, 1986); addition of antimycin A, oligomycin, valinomycin, potassium ascorbate, TMPD, and nucleoside phosphates; treatment with proteinase K or trypsin and addition of PMSF and soybean trypsin inhibitor; reisolation of mitochondria by centrifugation (Pfanner and Neupert, 1986, 1987b); assessment of import of ADP/ATP carrier by hydroxylapatite chromatography (Schleyer and Neupert, 1984; Pfanner and Neupert, 1987b); immunoprecipitation of ADP/ATP carrier, $F_1\beta$, and ATPase subunit 9 and SDS-polyacrylamide gel electrophoresis (Schleyer et al., 1982; Pfanner and Neupert, 1985); fluorography (Chamberlain, 1979) and quantitation of the fluorograms by densitometry (Pfanner and Neupert, 1985).

The reaction volume for binding and import of precursors at 25°C was 200 μl, including a buffer containing 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS and 3% (w/v) BSA, adjusted to pH 7.2 with KOH. The amounts of mitochondria and reticulocyte lysate added were such that the samples contained 25 μg of mitochondrial protein and 75 μl of reticulocyte lysate. All reactions were made chemically identical by adding the same volume of reagent-free solvent to the control samples. After the binding and import reactions, the reisolated mitochondria were washed in SEM medium or in the described BSA-containing buffer and transferred to new sample tubes. In some experiments, the wash buffers contained valinomycin and apyrase (0.2 U/ml). Each experiment was performed at least 3 times; the variations between the experiments were less than 10%.

Construction of Fusion Proteins
Fusion Protein pSu9$_{1-69}$-DHFR
The vector used was pDS5/2, which contains the cDNA coding for mouse DHFR downstream of a T5 promoter (Stueber et al., 1984). It was linearized at the unique BamHI site in front of the DHFR gene and treated with S1 nuclease, Klenow polymerase, and calf intestinal phosphatase (Maniatis et al., 1982). A full-length cDNA coding for subunit 9 of F_0-ATPase was isolated from a N. crassa library constructed according to Gubler and Hoffman (1983). According to the published sequence of subunit 9 cDNA (Viebrock et al., 1982), it contains two Sau3A sites, one of which cleaves in the triplet coding for glutamic acid—the fourth amino acid of the mature subunit 9 (Sebald et al., 1980). The cDNA was cut with Sau3A, and a 280 bp PstI-Sau3A piece, which contained the coding region for the entire presequence plus 5'-untranslated region, was isolated, treated with S1 nuclease and Klenow polymerase, and ligated into a pDS5/2 vector that was treated as described above. Transformation of E. coli strain DH1 was done according to Hanahan (1983). Plasmid DNAs of the resulting clones, which had EcoRI-HindIII inserts of about 950 bp (280 bp from the presequence plus approximately 670 bp from the DHFR cDNA), were sequenced according to Chen and Seeburg (1985), using a synthetic DHFR specific primer, 5'-CCATATTTTGGGACAC-3', corresponding to nucleotides 48–32 of the antisense strand of the coding region of DHFR. The amino acid sequence at the junction between the subunit 9 presequence and DHFR reads FQKRAYSSdgiMVRP (amino acids in capital letters correspond to subunit 9 [left] or DHFR sequences [right] separated by the amino acids introduced by cloning [lower case]; Y is the first amino acid of the mature subunit 9 [Sebald et al., 1980]). Transcription with E. coli RNA polymerase, capping, and translation of the resulting RNAs were done as described (Stueber et al., 1984). For the in vitro import reactions, "no synthesis lysate" was included such that each sample contained 75 μl of reticulocyte lysate.

Fusion Protein pSu9$_{1-147}$-DHFR
The cDNA coding for subunit 9 (see above) was inserted in the correct orientation into the PstI site of pDS5 (Stueber et al., 1984), resulting in plasmid pI16/23. Plasmid pI16/23 was treated with NsiI, which cuts 5 nucleotides downstream of the TAA stop codon of subunit 9 (Viebrock et al., 1982), resulting in a 3'-overhanging piece of 4 nucleotides. We also used a high concentration of S1 nuclease (5 U per μg

of DNA) to resect a piece of double-stranded DNA into the stop codon. Afterwards, the plasmid was treated with Klenow polymerase and calf intestinal phosphatase. DHFR cDNA was cut out of pDS5/2 as a BamHI–HindIII fragment, treated with S1 nuclease and Klenow polymerase, and blunt end–ligated into the vector described above. Correct orientation of the DHFR cDNA and the sequence of the junction were determined by double-stranded DNA sequencing, using the DHFR-specific primer mentioned above. The amino acid sequence around the junction reads AKFTygiMVRP (amino acids in capital letters correspond to the C-terminal part of the subunit 9 [left] and the N-terminal part of DHFR [right]; the TAA stop codon of subunit 9 was changed by the cloning procedure into a TAC tyrosine codon; amino acids in lower case were introduced by the cloning procedure).

Acknowledgments

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