Transport of F_1-ATPase subunit β into mitochondria depends on both a membrane potential and nucleoside triphosphates

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Transport of cytoplasmically synthesized precursor proteins into or across the inner mitochondrial membrane requires a mitochondrial membrane potential. We have studied whether additional energy sources are also necessary for protein translocation. Reticulocyte lysate (containing radiolabelled precursor proteins) and mitochondria were depleted of ATP by pre-incubation with apyrase. A membrane potential was then established by the addition of substrates of the electron transport chain. Oligomycin was included to prevent dissipation of $\Delta\psi$ by the action of the F_0F_1-ATPase. Under these conditions, import of subunit β of F_1-ATPase ($F_1\beta$) was inhibited. Addition of ATP or GTP restored import. When the membrane potential was destroyed, however, the import of $F_1\beta$ was completely inhibited even in the presence of ATP. We therefore conclude that the import of $F_1\beta$ depends on both nucleoside triphosphates and a membrane potential.

Mitochondria *Protein import* *Membrane potential* *ATP* *F_1-ATPase*

1. INTRODUCTION

The vast majority of mitochondrial proteins are synthesized on cytoplasmic polysomes and are subsequently imported into mitochondria [1–3]. Transport of precursor proteins into or across the inner membrane requires energy [4–6]. Energization of the inner membrane has been shown to be essential [7–9]; the energy form necessary is the electrical component $\Delta\psi$ of the total protonmotive force [10,11]. In all these studies, however, it could never be excluded that in addition to $\Delta\psi$, high energy phosphate compounds, e.g. ATP, were also necessary.

Here we report on experiments in which the membrane potential and the level of ATP were manipulated independently of each other. Import of subunit β of the F_0F_1-ATPase ($F_1\beta$) into mitochondria was found to occur only when both a membrane potential and nucleoside triphosphates were present.

2. MATERIALS AND METHODS

Growth of *Neurospora crassa* (wild-type 74A) [7] and isolation of mitochondria by differential centrifugation [10] or by Percoll density gradient centrifugation [12] were carried out as described, except that the time for grinding of hyphae was reduced to 30–60 s and that the buffer for the Percoll gradient contained 30% (v/v) Percoll (Pharmacia), 200 mM sucrose, 80 mM KCl, 1 mM EDTA, 10 mM Mops and 3% (w/v) bovine serum albumin (BSA), adjusted to pH 7.2 with KOH. Mitochondria were washed in SEM medium (250 mM sucrose, 1 mM EDTA, 10 mM Mops, adjusted to pH 7.2 with KOH) and resuspended in SEM at a protein concentration of 2.5 mg/ml.

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Precursor proteins were synthesized in vitro in a rabbit reticulocyte lysate [13] programmed with *Neurospora* poly(A)$^+$ RNA in the presence of [^{35}S]methionine. Postribosomal supernatants were prepared and supplemented as previously described [7].

Apyrase (Sigma A6160, grade VIII; approx. 250 units per mg protein; units are based on ATPase activity) was dissolved in water at 1 unit/μl and, when necesssary, diluted further with water. Labelled reticulocyte lysate (75 μl) was incubated with apyrase for 15 min at 30°C and 15 min at 25°C. The mitochondrial suspension (75 μl at 2.5 mg/ml in SEM) was incubated with apyrase for 15 min at 25°C. The amounts of apyrase are given in the figure legends. Controls received either water or apyrase which had been boiled for 10 min. After cooling to 0°C 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, was added to the reticulocyte lysate. Antimycin A and oligomycin (8 μM and 20 μM final concentrations, respectively) were added from a 100-fold concentrated stock solution in ethanol. Potassium ascorbate (pH 7) and *N,N,N',N'*-tetramethylphenylenediamine (TMPD) (8 mM and 0.2 mM final concentrations, respectively) were added from 50-fold concentrated solutions in water. Mitochondria, corresponding to 25 μg mitochondrial protein, were then added. ATP (Boehringer), GTP (Boehringer), adenosine 5'-(β,γ-methylene)triphosphate (AMP-PCP) (Sigma) and adenosine 5'-(β,γ-imido)-triphosphate (AMP-PNP) (Sigma) were prepared as 200 mM solutions in water and either neutralized with KOH just before use, or sufficient amounts of Mops, pH 7.2, were included in the reaction mixture such that the final reaction mixture was neutral. All reactions were made chemically identical by adding the same volume of reagent free solvent to the control samples. The final reaction volume was 200 μl. Samples were incubated for 25 min at 25°C. After addition of PMSF (1 mM final concentration), mitochondria were reisolated by centrifugation for 15 min at 27000 × *g*. For protease treatment, samples were cooled to 0°C after the 25 min 25°C incubation and proteinase K (Boehringer) in SEM was added to a final concentration of 15 μg/ml. After 25 min at 0°C, PMSF (1 mM final concentration) was

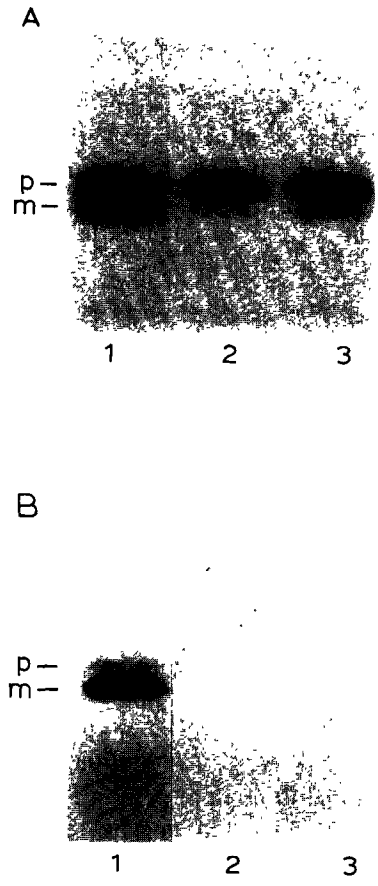

Fig.1. Preincubation of reticulocyte lysate and mitochondria with apyrase inhibits the import of $F_1\beta$. Isolated mitochondria and reticulocyte lysate (containing radiolabelled precursor proteins) were preincubated with the following: apyrase (corresponding to 6 units) which had been heated to 95°C for 10 min (lane 1); 6 units apyrase (lane 2); 0.025 units apyrase (lane 3). The mitochondria were added to the reticulocyte lysate in a mixture containing antimycin A, oligomycin, ascorbate, TMPD and a BSA containing buffer (for details see section 2). After 25 min at 25°C, mitochondria were reisolated and $F_1\beta$ was immunoprecipitated. The samples were then resolved by SDS-polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown (A). Experiment B was performed as described for A except that the samples were treated with proteinase K (see section 2) after the 25 min 25°C incubation but prior to the reisolation of the mitochondria. When water was used instead of boiled apyrase for the preincubation, the same result was obtained as shown in lanes 1. p, precursor of $F_1\beta$; m, mature form of $F_1\beta$.

added, and samples were incubated for 10 min further at 0°C. Mitochondria were then reisolated as described above.

Published procedures were used for immunoprecipitation with specific antiserum directed against $F_1\beta$ using protein A-Sepharose, for SDS-polyacrylamide gel electrophoresis [7,10,14] and fluorography [15]. Mitochondrial protein was determined according to [16].

3. RESULTS

Isolated *Neurospora* mitochondria and rabbit reticulocyte lysate containing radiolabelled precursor proteins were incubated with apyrase (an adenosine 5′-triphosphatase and an adenosine 5′-diphosphatase) to deplete them of endogenous ATP (and ADP). Then, the mitochondria were added to the reticulocyte lysate. The reaction mixture contained ascorbate plus TMPD, which supplies electrons at the level of complex IV to the electron transport chain, and antimycin A and oligomycin which inhibit complex III and the F_0F_1-ATPase, respectively [7,17]. Under these conditions the membrane potential created by ascorbate plus TMPD could not be reduced by the activity of the F_0F_1-ATPase. Samples were incubated for 25 min at 25°C. One set of samples (experiment B) was then treated with proteinase K to digest $F_1\beta$ precursor which had not been imported. Mitochondria were reisolated from all samples. $F_1\beta$ was immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis. Fluorograms of the dried gels are shown in fig.1. Controls (which received apyrase heated before use) show processing of the precursor (p) of $F_1\beta$ to the mature form (m) which is catalyzed by the matrix-localized processing peptidase (fig.1A, lane 1) and protection from externally added protease of mature $F_1\beta$ (fig.1B, lane 1). Thus, the criteria for import and processing of $F_1\beta$ [14,18] were fulfilled. When lysate and mitochondria were pretreated with apyrase, import and processing of $F_1\beta$ were inhibited (fig.1A and B, lanes 2 and 3). In control experiments it was demonstrated that the protease resistance of endogenous $F_1\beta$ and the amount of precursor proteins in the reticulocyte lysate were not affected by pre-treatment with apyrase (not shown). Therefore, it can be concluded that pre-treatment with apyrase inhibits import of $F_1\beta$ into mitochondria.

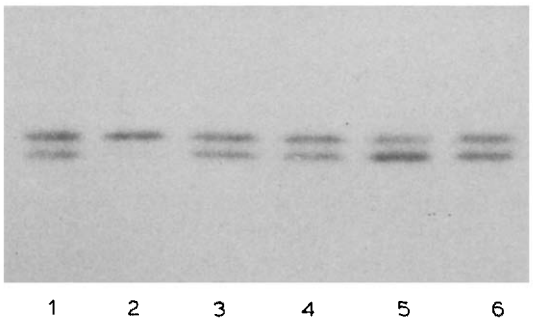

Fig.2. ATP and GTP restore the specific processing of $F_1\beta$ after pre-treatment with apyrase. The experiment was performed as described in the legend to fig.1A with the following modifications. Samples 2–6 were pretreated with 0.03 units apyrase and sample 1 with the same amount of boiled apyrase. 4 mM ATP (lane 3) and 8 mM ATP (lane 4), 4 mM GTP (lane 5) and 8 mM GTP (lane 6) were included in the import reaction (25 min, 25°C). Half of the total amount of the nucleotides was added at the beginning of this incubation, the other half was added in the middle of the incubation period.

When ATP was included in the reaction mixture, specific processing (fig.2, lanes 3 and 4) and

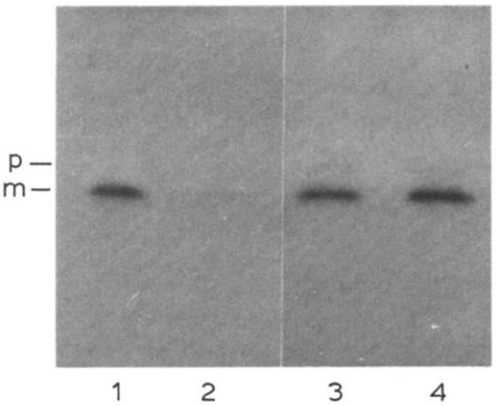

Fig.3. ATP and GTP restore the transport of $F_1\beta$ into protease inaccessible location. The experiment was performed as described in the legends to figs 1B and 2 with the following modifications. Samples 2–4 were pretreated with 0.03 units apyrase and sample 1 with the same amount of boiled apyrase. 6 mM ATP (lane 3) and 6 mM GTP (lane 4) were included in the import reaction (25 min, 25°C).

protease protection (fig.3, lane 3) of $F_1\beta$ were restored. Similarly, GTP restored import of $F_1\beta$ (fig.2, lanes 5 and 6, and fig.3, lane 4). This restoration was not achieved by addition of the ATP analogues AMP-PCP or AMP-PNP (not shown). Since nucleoside phosphate kinases are present in mitochondria, it cannot be determined from these studies which form of high energy phosphate compounds is required.

In the following experiments it was confirmed that nucleoside triphosphates cannot substitute for the requirement for a membrane potential. For this purpose, the treatment with apyrase was omitted. The mitochondrial membrane potential was destroyed by addition of antimycin A and oligomycin [7,10,17]. Under these conditions, specific processing (fig.4A, lane 2) and protease protection (fig.4B, lane 2) of $F_1\beta$ were inhibited. Addition of ATP did not restore import (fig.4A and B, lanes 3 and 4). The import of $F_1\beta$ occurred only when a membrane potential was established by either adding ascorbate plus TMPD (fig.4A and B, lane 1) or by omitting oligomycin in the presence of ATP and thereby allowing the formation of a membrane potential via the F_0F_1-ATPase (fig.4A and B, lanes 5 and 6).

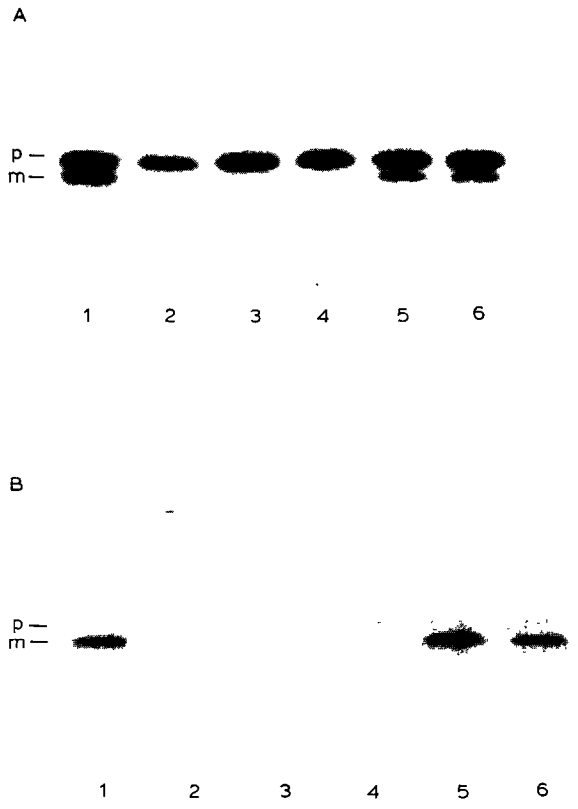

Fig.4. ATP cannot substitute for the requirement of a membrane potential. The experiments were performed as described in the legends to figs 1 and 2 with the following modifications. Pre-treatment with apyrase was omitted. All samples contained antimycin A. Samples 1–4 contained oligomycin. Sample 1 received ascorbate plus TMPD. Samples 3 and 5 received 4 mM ATP. Samples 4 and 6 received 8 mM ATP.

4. DISCUSSION

The results presented here demonstrate that the in vitro import of a mitochondrial precursor protein is dependent on the presence of nucleoside triphosphates, e.g. ATP, in addition to a membrane potential. Since the system used contains nucleoside phosphate kinases, it cannot yet be decided which form of high energy phosphate compounds is necessary. Further studies have to be undertaken in order to clarify whether other mitochondrial precursor proteins show the same requirements and to investigate the specific role played by the high energy phosphate compounds. Their function could involve conformational stabilization of precursor proteins, or they could be required at the level of binding of precursors to receptor sites on the mitochondrial surface or at the level of translocation across the mitochondrial membranes. A role in proteolytic processing per se seems unlikely since virtually no protease protected precursor [14] was found after pre-treatment with apyrase. The existence of at least three different import pathways for mitochondrial precursor proteins has been shown [6,19]; thus, the requirement for high energy phosphate compounds could be restricted to certain classes of precursor proteins.

A requirement for both ATP and a membrane potential has recently been reported for protein transport in *Escherichia coli* [20]. Earlier studies mainly emphasized the role of an electrochemical potential [21–26], one group, however, has reported a requirement for ATP but not an essential requirement for a membrane potential [27]. Interestingly, protein translocation into chloroplasts [28,29] and across the membrane of the endoplasmic reticulum [30–34] have been found to require ATP. In both these cases, however, an ad-

ditional requirement for a membrane potential could not be demonstrated.

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