Transport of proteins into mitochondria: a potassium diffusion potential is able to drive the import of ADP/ATP carrier

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The transfer of cytoplasmically synthesized precursor proteins into or across the inner mitochondrial membrane is dependent on energization of the membrane. To investigate the role of this energy requirement, a buffer system was developed in which efficient import of ADP/ATP carrier into mitochondria from the receptor-bound state occurred. This import was rapid and was dependent on divalent cations, whereas the binding of precursor proteins to the mitochondrial surface was slow and was independent of added divalent cations. Using this buffer system, the import of ADP/ATP carrier could be driven by a valinomycin-induced potassium diffusion potential. The protonophore carbonylcyanide *m*-chlorophenylhydrazone was not able to abolish this import. Imposition of a ΔpH did not stimulate the import. We conclude that the membrane potential $\Delta\psi$ itself and not the total protonmotive force Δp is the required energy source.

Key words: ADP/ATP carrier/membrane potential/mitochondria/potassium diffusion potential/protein import

Introduction

Most mitochondrial proteins are encoded by nuclear genes and synthesized on free cytoplasmic polysomes as water-soluble precursors (for review, see Hay *et al.*, 1984; Harmey and Neupert, 1985). In many cases these precursors carry amino-terminal peptide extensions. The precursor proteins bind to proteinaceous receptors on the mitochondrial surface (Hennig *et al.*, 1983; Zwizinski *et al.*, 1984) and are then translocated across the membranes. Import into or across the inner membrane requires an energized membrane (Schleyer *et al.*, 1982; Gasser *et al.*, 1982; Kolanski *et al.*, 1982). During or after the import the amino-terminal peptide extension is removed by a matrix-located processing peptidase (Böhni *et al.*, 1980; Conboy *et al.*, 1982; Schmidt *et al.*, 1984).

The role of an energy requirement for the import of proteins into mitochondria is unknown. In order to understand this role it is essential to know whether the total protonmotive force Δp (with the components membrane potential $\Delta\psi$ and pH gradient ΔpH) or the membrane potential $\Delta\psi$ alone is required. To study this problem it is necessary to be able to manipulate ΔpH and $\Delta\psi$ without disturbing the import of precursor proteins.

We investigated the *in vitro* import of the ADP/ATP carrier into isolated mitochondria of the fungus *Neurospora crassa*. The functional form of the ADP/ATP carrier in the inner mitochondrial membrane is a dimer of two identical subunits (mol. wt. 32 000) (Klingenberg *et al.*, 1978). The precursor of the ADP/ATP carrier is synthesized with the same apparent mol. wt. as displayed by the mature protein (Zimmermann and Neupert, 1980). After synthesis in a cell-free translation system (rabbit reticulocyte lysate) the ADP/ATP carrier becomes associated with added isolated mitochondria. If the mitochondria are de-energized, the ADP/ATP carrier binds to receptors on the mitochondrial surface (Zwizinski *et al.*, 1983, 1984). In this location, the precursor is very sensitive to externally added proteases. After re-energization of the mitochondria the carrier is imported into a protease-protected location and acquires properties of the carrier imported *in vivo* (e.g., binding of the specific inhibitor carboxyatractyloside) (Schleyer and Neupert, 1984).

We describe here a buffer system in which the ADP/ATP carrier can be imported from the receptor-bound state with the same efficiency as from reticulocyte lysate. Using this buffer, the import of carrier can be driven by a valinomycin-induced potassium diffusion potential. Imposing a ΔpH does not affect the import, and the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) cannot abolish the import driven by a potassium diffusion potential. We suggest that the membrane potential itself is the required energy form for import and not a movement of protons driven by the total protonmotive force.

Results

A buffer system for import of precursor proteins

As a prerequisite for studying the energy requirements of import it was necessary to develop a simple buffer system in which transmembrane movement of precursor proteins can be followed. A buffer (pH 7.2) containing 0.25 M sucrose, 70 – 100 mM KCl or NaCl, 5 mM $MgCl_2$, 3% bovine serum albumin (BSA) and 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) was found to be adequate ('import buffer'). Reticulocyte lysate was diluted up to 120-fold with this 'import buffer'. Import of ADP/ATP carrier into isolated mitochondria was assayed by analysing the acquisition of protease resistance; binding was assayed by determining the association of the precursor to ADP/ATP carrier with mitochondria in the presence of antimycin A/oligomycin which block complex III of the respiratory chain and the H^+-translocating ATPase, respectively (Schleyer *et al.*, 1982; Nicholls, 1982; Wikstrom and Krab, 1982). Under these conditions binding and import of carrier were even slightly higher than in undiluted lysate. Up to a dilution of 20-fold the same level of import was observed without energy substrates being added, presumably because of the substrates already present in the reticulocyte lysate. At higher dilutions the addition of substrates [e.g., ascorbate and N,N,N′,N′-tetramethylphenylenediamine (TMPD) which lead to injection of electrons at the level of complex IV (Schleyer *et al.*, 1982; Nicholls, 1982; Wikstrom and Krab, 1982)] was necessary (Figure 1A).

When 1 mM EDTA was added to diluted (20- to 120-fold) lysates in which $MgCl_2$ was omitted, binding of precursor to mitochondria was not affected (Figure 1B, lane 2), but import was diminished. At dilutions of 60-fold and higher import was completely inhibited (Figure 1B, lane 4). Addition of $MgCl_2$ reversed the effect of EDTA (Figure 1B, lane 3). The concentration of

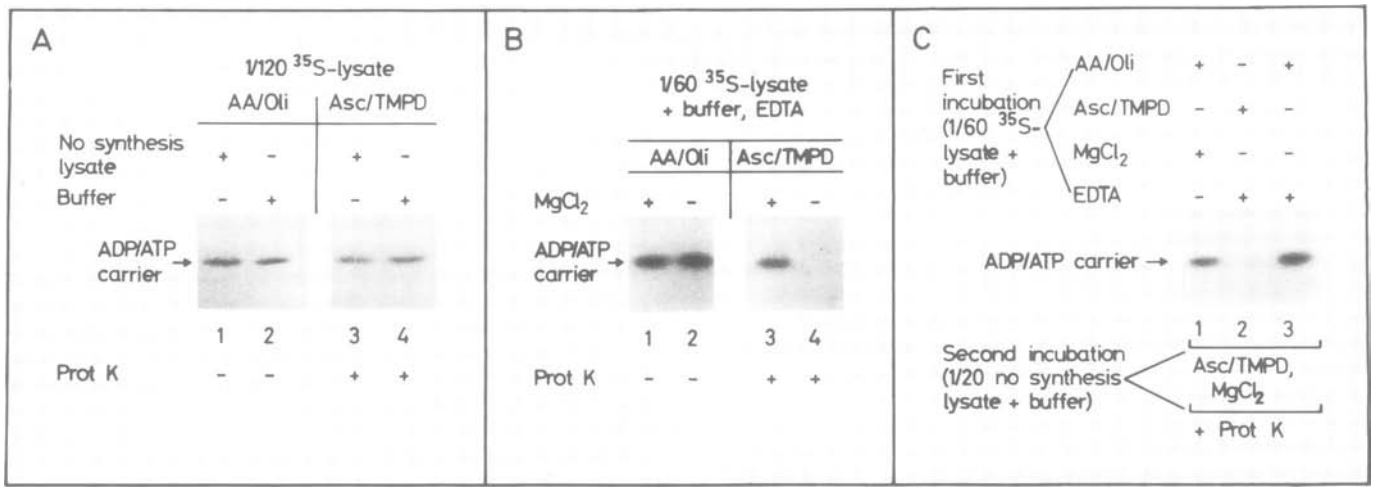

Fig. 1. Import of ADP/ATP carrier from reticulocyte lysate diluted with buffer. **(A)** Comparison of binding and import of ADP/ATP carrier from undiluted and diluted lysate. Freshly isolated *Neurospora* mitochondria (25 μg protein) were mixed with 6 μl rabbit reticulocyte lysate containing ^{35}S-labelled *Neurospora* proteins ('^{35}S-lysate'). Reactions **(1)** and **(2)** contained 4 μM antimycin A (AA) and 10 μM oligomycin (Oli), reactions **(3)** and **(4)** contained 4 mM potassium ascorbate (Asc) and 0.2 mM TMPD. Reactions **(1)** and **(3)** contained 'no synthesis lysate' (see Materials and methods) and reactions **(2)** and **(4)** contained buffer (final volume 720 μl). The buffer was 0.25 M sucrose, 80 mM KCl, 5 mM $MgCl_2$, 3% (w/v) BSA and 10 mM MOPS, adjusted to pH 7.2 with KOH. Samples were incubated for 30 min at 25°C. After 15 min at 0°C reactions **(1)** and **(2)** received SEM (see Materials and methods) and reactions **(3)** and **(4)** received proteinase K (Prot K) in SEM to a final concentration of 20 μg/ml. After 30 min at 4°C PMSF was added to all samples to a final concentration of 1 mM and incubation was continued for another 5 min at 4°C. Mitochondria were re-isolated, solubilized in Triton X-100-containing buffer, immunoprecipitated for ADP/ATP carrier and precipitates were subjected to SDS-polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown. **(B)** Divalent ions are necessary for import, but not for binding. Isolated *Neurospora* mitochondria (25 μg protein) were mixed with 10 μl ^{35}S-labelled reticulocyte lysate; Reactions **(1)** and **(2)** contained 4 μM antimycin A and 10 μM oligomycin, reactions **(3)** and **(4)** contained 4 mM potassium ascorbate and 0.2 mM TMPD. Reactions **(1)** and **(3)** received $MgCl_2$ to a final concentration of 5 mM. All samples contained buffer to a final volume of 600 μl. The buffer was 0.25 M sucrose, 70 mM KCl, 1 mM EDTA, 3% (w/v) BSA and 10 mM MOPS, adjusted to pH 7.2 with KOH. Samples were incubated for 30 min at 25°C. Total ADP/ATP carrier [reactions **(1)** and **(2)**] and protease-resistant carrier [reactions **(3)** and **(4)**] associated with mitochondria were determined as described in **(A)**. **(C)** Binding to de-energized mitochondria in the absence of divalent cations is specific. Isolated *Neurospora* mitochondria (25 μg protein) were mixed with 10 μl ^{35}S-labelled reticulocyte lysate. Reactions **(1)** and **(3)** contained 4 μM antimycin A and 10 μM oligomycin, reaction **(2)** contained 4 mM potassium ascorbate and 0.2 mM TMPD. All reactions contained buffer to a final volume of 600 μl. The buffer was 0.25 M sucrose, 70 mM KCl, 3% (w/v) BSA and 10 mM MOPS, adjusted to pH 7.2 with KOH. Reaction **(1)** contained 5 mM $MgCl_2$ and reactions **(2)** and **(3)** contained 1 mM EDTA. Samples were incubated for 30 min at 25°C and cooled to 0°C. Mitochondria were re-isolated and each sample was resuspended in 400 μl of a mixture containing buffer [0.25 M sucrose, 70 mM KCl, 5 mM $MgCl_2$, 3% (w/v) BSA and 10 mM MOPS, adjusted to pH 7.2 with KOH], 4 μM antimycin A, 10 μM oligomycin and 20 μl 'no synthesis lysate'. Each sample received 4 mM potassium ascorbate and 0.2 mM TMPD and was incubated for 15 min at 25°C. After 15 min at 0°C all samples were assessed for protease-resistant ADP/ATP carrier associated with mitochondria as described in **(A)**.

$MgCl_2$ for optimal import was in the range of 3–5 mM. To analyse whether binding without divalent cations is specific, the precursor of the ADP/ATP carrier was bound to de-energized mitochondria in the presence of EDTA. Mitochondria were re-isolated, then $MgCl_2$ was added and the mitochondria were re-energized by addition of ascorbate and TMPD. Import occurred with the same efficiency as when binding was performed in the presence of divalent cations (Figure 1C, lanes 1 and 3). Apparently, binding to de-energized mitochondria in the presence of EDTA leads to a receptor-bound precursor which is competent for further import. These findings suggests that divalent cations are necessary for import, but not for binding.

In the absence of divalent cations ADP/ATP carrier was bound to energized mitochondria as efficiently as it was to de-energized mitochondria. However, import was found to be strongly reduced when mitochondria were re-isolated and re-energized in the presence of divalent cations (Figure 1C, lane 2). Probably, the precursor moves into a location which is not on the assembly pathway.

Import from the receptor-bound state can be performed in buffer

Radiolabelled ADP/ATP carrier was bound to de-energized mitochondria in reticulocyte lysate (addition of antimycin A and oligomycin). After re-isolation, the mitochondria were resuspended in 'import buffer' and re-energized with ascorbate and TMPD (Figure 2, reactions 3). Import occurred with the same efficiency as import after resuspension in 'no synthesis lysate' lacking mitochondrial precursor proteins (see Materials and methods) (Figure 2, reactions 2) (Zwizinksi *et al.*, 1983) or import of free precursor (Figure 2, reactions 1). Washing of mitochondria with buffer before import in buffer did not diminish binding or import (Figure 2, reactions 4). By using this simple buffer system it was now possible to study the translocation from the receptor-bound state in greater detail.

Kinetics of import in buffer

ADP/ATP carrier was bound to de-energized mitochondria (addition of antimycin A and oligomycin), the mitochondria were re-isolated and resuspended in 'import buffer' or 'no synthesis lysate'. For the times indicated in Figure 3, mitochondria were re-energized with ascorbate and TMPD, then the import was stopped with potassium cyanide which blocks complex IV of the respiratory chain (Schleyer *et al.*, 1982, Nicholls, 1982). Both in buffer (Figure 3) and in 'no synthesis lysate' (not shown) import of receptor-bound precursor occurred within 1 min.

Previous work in our laboratory has shown that binding of precursor from the reticulocyte lysate reached its maximal value only after 20–30 min at 25°C. Apparently, binding to the receptor occurs in a rather slow reaction, but directs the precursor into such a location that it can be rapidly translocated into the inner membrane.

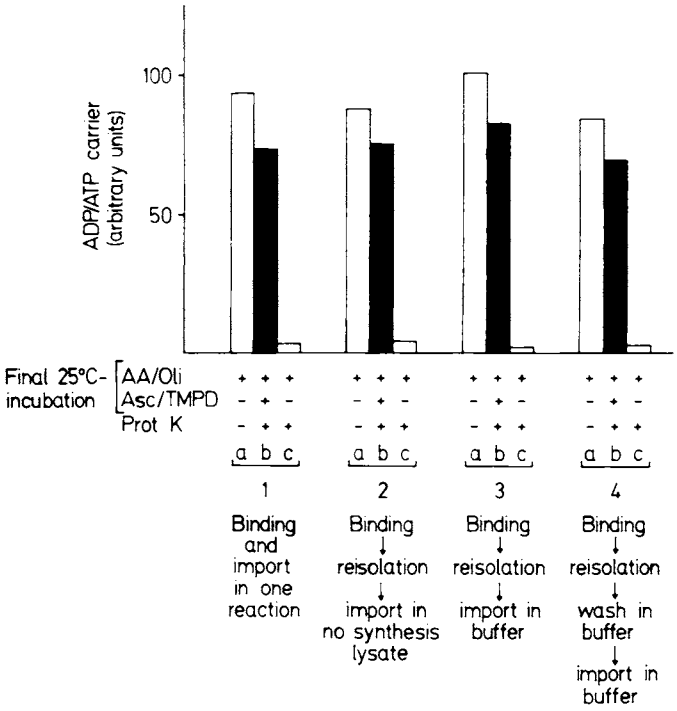

Fig. 2. Import from the receptor-bound state can be performed in buffer. Reactions contained 35 μl ^{35}S-labelled reticulocyte lysate, isolated *Neurospora* mitochondria (25 μg protein), 4 μM antimycin A and 10 μM oligomycin. To reaction **(1b)** 4 mM potassium ascorbate and 0.2 mM TMPD were added. Samples were incubated for 25 min at 25°C. After cooling to 0°C reaction **(1a)** was assessed for total ADP/ATP carrier and reactions **(1b)** and **(1c)** were assessed for protease-resistant carrier associated with mitochondria as described in the legend to Figure 1A. Here and in the following experiments results were quantitatively evaluated by densitometry of the X-ray films (see Materials and methods). Mitochondria of reactions **(2)** and **(3)** were re-isolated and re-suspended in 200 μl of 'no synthesis lysate' [reactions **(2)**] or buffer [0.25 M sucrose, 80 mM KCl, 5 mM $MgCl_2$, 3% (w/v) BSA and 10 mM MOPS, adjusted to pH 7.2 with KOH] [reactions **(3)**] which both contained 4 μM antimycin A and 10 μM oligomycin. Reactions **(2b)** and **(3b)** additionally contained 4 mM potassium ascorbate and 0.2 mM TMPD. After 15 min at 25°C and 15 min at 0°C, reactions were assessed for total carrier [**(2a)** and **(3a)**] and protease-resistant carrier [**(2b)**, **(2c)**, **(3b)** and **(3c)**] associated with mitochondria as described above. Mitochondria of reactions **(4)** were re-isolated, re-suspended at 0–4°C in the above described buffer containing 4 μM antimycin A and 10 μM oligomycin and again re-isolated. They were then treated as described for reactions **(3)**.

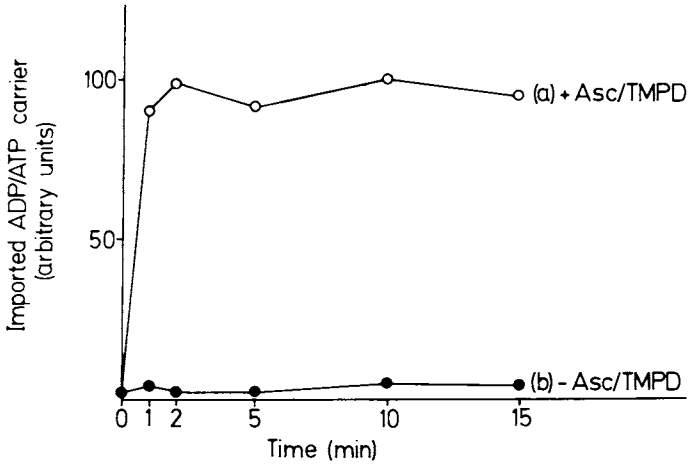

Fig. 3. Kinetics of import in buffer. 400 μl ^{35}S-labelled reticulocyte lysate received 4 μM antimycin A, 10 μM oligomycin and isolated *Neurospora* mitochondria (300 μg protein). Samples were incubated for 25 min at 25°C and cooled to 0°C. Mitochondria were re-isolated and re-suspended at 0°C in 120 μl buffer [0.25 M sucrose, 80 mM KCl, 5 mM $MgCl_2$, 3% (w/v) BSA and 10 mM MOPS, adjusted to pH 7.2 with KOH] containing 4 μM antimycin A and 10 μM oligomycin. Portions of 10 μl were transferred into 100 μl of buffer (composition as described above and pre-warmed to 25°C) containing antimycin A and oligomycin; reactions **(a)** also contained 4 mM potassium ascorbate and 0.2 mM TMPD. Reactions **(a)** were incubated for 1, 2, 5, 10 and 15 min at 25°C, reactions **(b)** for 0, 1, 2, 5, 10 and 15 min at 25°C. At the end of the respective incubation periods, samples were transferred into 500 μl of buffer at 0°C containing antimycin A/oligomycin and 1 mM potassium cyanide. After 15 min at 0°C samples were assessed for protease-resistant ADP/ATP carrier associated with mitochondria as described in the legends to Figures 1A and 2.

A potassium diffusion potential is able to drive the import of ADP/ATP carrier from the receptor-bound state

The ionophore valinomycin preferentially transports potassium ions (Nicholls, 1982; Reed, 1979). Re-suspension of mitochondria in a medium with a low potassium concentration in the presence of valinomycin and antimycin A/oligomycin (to inhibit the physiological membrane potential) leads to a potassium diffusion potential. This potential has the same orientation as the physiological membrane potential (positive outside) (Nicholls, 1974, 1982).

Radiolabelled ADP/ATP carrier was bound to de-energized mitochondria [antimycin A/oligomycin plus valinomycin; potassium concentration in reticulocyte lysate was ~90–100 mM (Schleyer *et al.*, 1982)]. After re-isolation, the mitochondria were resuspended in 'import buffer' with different KCl concentrations which contained antimycin A/oligomycin and valinomycin and which had been warmed to 25°C (Figure 4). NaCl was present such that each sample had the same concentration of monovalent ions. The import of ADP/ATP carrier increased with decreasing potassium concentration (Figure 4A) and exhibited essentially a linear dependence on the logarithm of the potassium concentration (Figure 4B). We conclude that this import is driven by a potassium diffusion potential.

These results were not due to the possible artifact that under the particular conditions of the experiment the imported ADP/ATP carrier had an altered protease sensitivity, since: (i) re-isolation of the mitochondria after incubation in the presence of a diffusion potential and protease treatment in 'import buffer' with 100 mM KCl and valinomycin plus antimycin A/oligomycin yielded the same result; (ii) the protease resistance of the endogenous (i.e., *in vivo* imported) ADP/ATP carrier was not diminished at higher KCl concentrations (data not shown).

De-salted radiolabelled reticulocyte lysates were diluted with buffer and the potassium concentrations were adjusted to values between 0.1 and 100 mM. Addition of ascorbate/TMPD and incubation for 30 min at 25°C led to the same level of import at all potassium concentrations; this level was comparable with that observed with untreated lysates. However, when valinomycin and antimycin A/oligomycin were added instead of ascorbate/TMPD, virtually no import was observed (not shown). As the binding of the precursor proteins is much slower than the import, it seems that the potassium diffusion potential is not maintained for the time needed to bind sufficient amounts of precursor to the receptor.

Of all precursor proteins which were tested, only the ADP/ATP carrier (see Figure 2) displayed the same efficiency of import from lysate as from the receptor-bound state. Other precursor proteins which need an energized inner membrane for import (e.g., subunit 2 and subunit 9 of the F_0F_1-ATPase, cytochrome

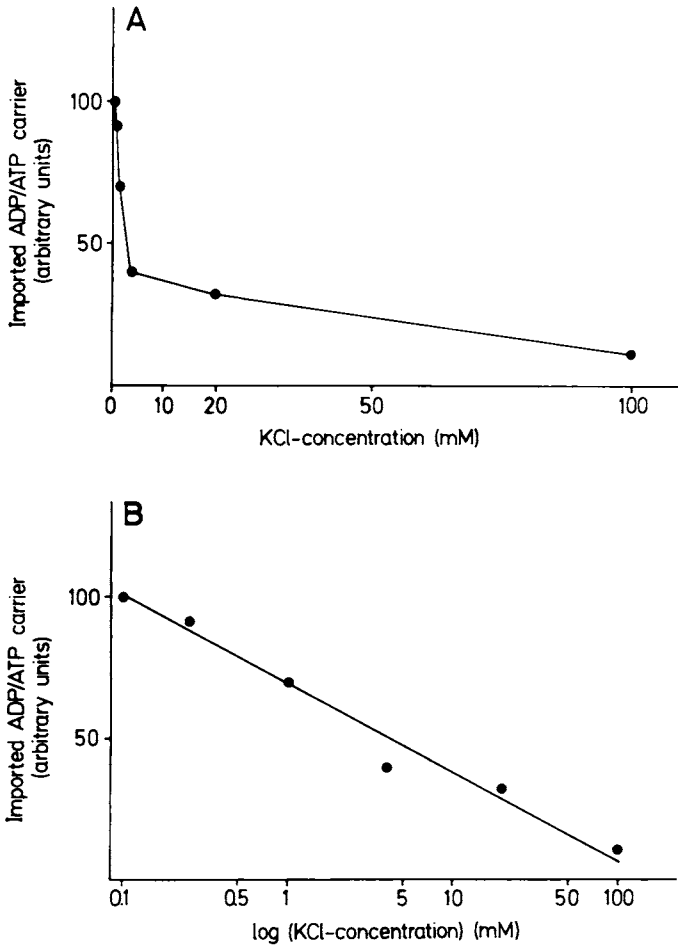

Fig. 4. Import driven by a potassium diffusion potential. Samples containing 35 μl ^{35}S-labelled reticulocyte lysate, isolated mitochondria (25 μg protein), 0.1 μM valinomycin, 4 μM antimycin A and 10 μM oligomycin, were incubated for 25 min at 25°C. After cooling to 0°C, mitochondria were re-isolated and re-suspended in 300 μl buffer (pre-warmed to 25°C) containing valinomycin, antimycin A and oligomycin at the same concentrations as mentioned above. The buffer was 0.25 M sucrose, KCl and NaCl at a total concentration of 100 mM, 5 mM $MgCl_2$, 3% (w/v) BSA and 10 mM MOPS, adjusted to pH 7.2 with NaOH; KCl concentrations were 0.1, 0.25, 1, 4, 20 and 100 mM, respectively. After 3 min at 25°C and 15 min at 0°C samples were assessed for protease-resistant ADP/ATP carrier associated with mitochondria. The KCl concentrations at the final 25°C incubation are plotted on a linear scale in (**A**) and on a logarithmic scale in (**B**).

c_1 and subunit V of the bc_1-complex) (Teintze and Neupert, 1983) were not efficiently imported when first bound to de-energized mitochondria. Therefore import by a potassium diffusion potential could not be analysed with these proteins.

Imposing a ΔpH does not stimulate import of the receptor-bound precursor

Import of receptor-bound radiolabelled ADP/ATP carrier was analysed at external pH values between 6.0 and 7.5, at a fixed low potassium diffusion potential, by resuspending mitochondria in the appropriate 'import buffers' (Figure 5). Import kinetics in these experiments were comparable with those described in Figure 3. Higher external proton concentrations did not lead to higher import (Figure 5). Assuming that a ΔpH is stable for the time which is required to obtain import from the receptor-bound state by a diffusion potential, we conclude that generation of a ΔpH does not promote import.

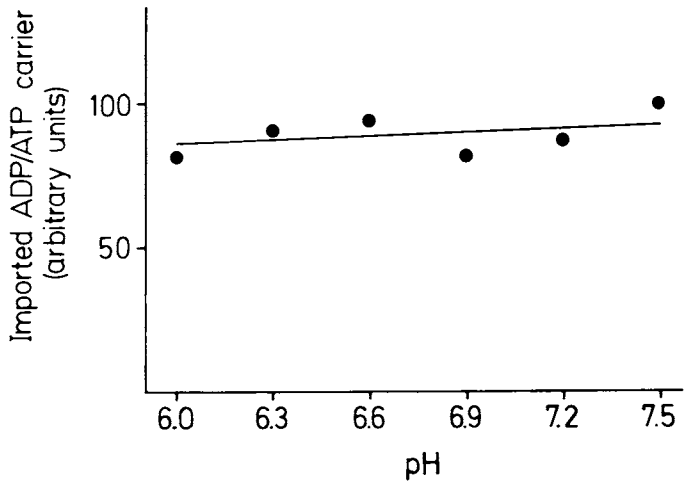

Fig. 5. Import driven by a potassium diffusion potential at different external pH values. The experiment was performed as described in the legend to Figure 4 with the following modifications. The buffer was 0.22 M sucrose, 4 mM KCl, 96 mM NaCl, 5 mM $MgCl_2$, 3% (w/v) BSA and 15 mM MOPS, at the indicated pH values (adjusted with NaOH or HCl).

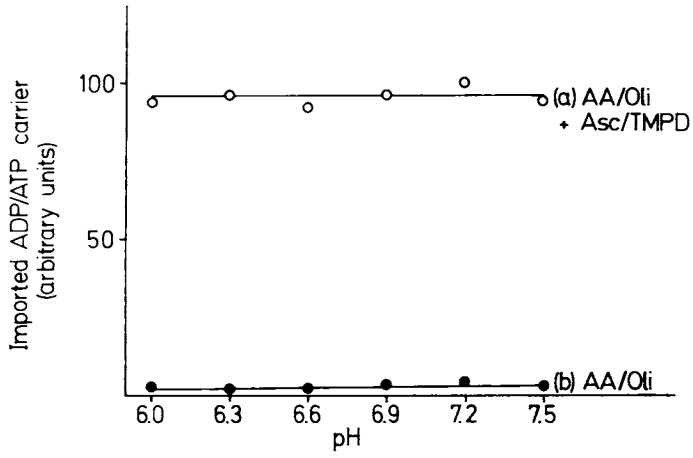

Fig. 6. Import driven by a proton potential at different external pH values. The experiment was performed as described in the legend to Figure 5 with the following modifications. Valinomycin was omitted from all samples. The buffer contained 100 mM KCl (no NaCl) and reactions (**a**) contained 4 mM potassium ascorbate and 0.2 mM TMPD.

In the absence of valinomycin but presence of antimycin A/oligomycin, imposing a ΔpH did not lead to higher import, either (Figure 6, reactions b). Thus it seems unlikely that a possible effect of ΔpH on import was obscured by a H^+/K^+ exchange; such an exchange may take place in mitochondria treated with valinomycin (Azzone *et al.*, 1978). Import of receptor-bound ADP/ATP carrier which was driven by ascorbate/TMPD also did not show a dependence on external pH (Figure 6, reactions a). Apparently, inactivation of import components by low pH did not occur.

The protonophore CCCP cannot abolish the import driven by a potassium diffusion potential

The protonophore CCCP (Heytler, 1979; Nicholls, 1982) completely inhibits the import driven by a proton potential both in lysate (Schleyer *et al.*, 1982) and in buffer (not shown). On the other hand, CCCP decreased the import driven by a valinomycin-induced potassium diffusion potential only to a limited degree, even at excessively high concentrations of 150 μM (Figure 7A).

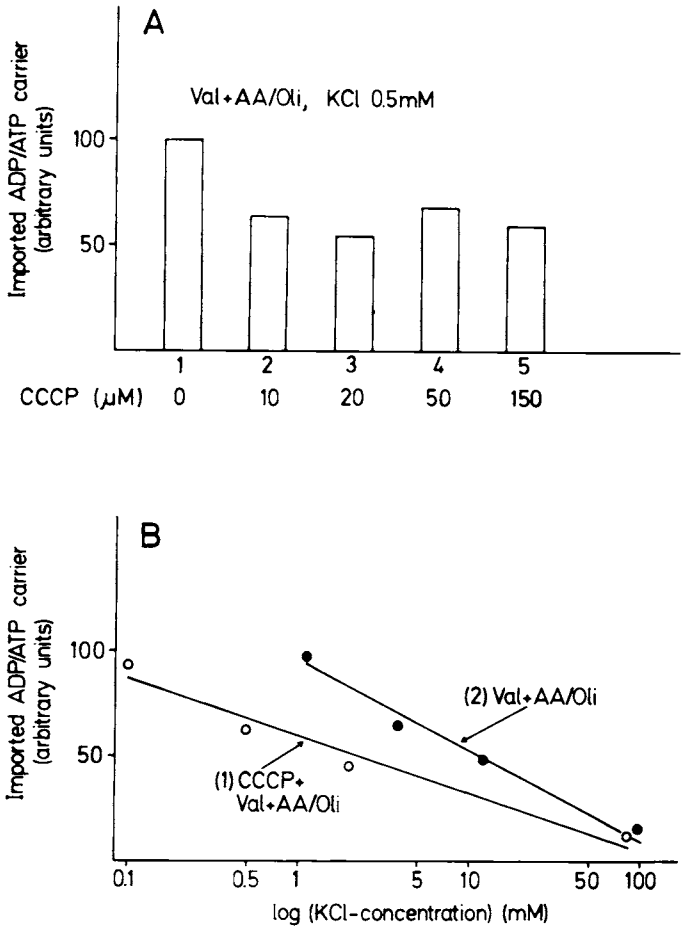

Fig. 7. CCCP does not abolish the import driven by a potassium diffusion potential. Experiments were performed as described in the legend to Figure 4 with the following modifications. **(A)** Reactions **(2)** – **(5)** contained CCCP at the final concentrations of 10, 20, 50 and 150 μM in both 25°C incubations, respectively. The buffer contained 0.5 mM KCl. **(B)** Reactions **(1)** contained 50 μM CCCP. The KCl concentrations of the buffer are indicated in the figure.

In the latter situation, essentially all protons driven by the total protonmotive force can be assumed to be transported by CCCP. Since import of proteins continues, a Δp-dependent transmembrane movement of protons cannot play an essential role in protein import.

In order to explain this moderate decrease of the import in the presence of CCCP (Figure 7A, lanes 2 – 5) the following experiment was carried out. Import driven by a potassium diffusion potential was measured in the absence or presence of CCCP at varying external KCl concentrations (Figure 7B). In the presence of CCCP lower KCl concentrations were required to obtain the same level of import as in the absence of CCCP. For instance, the same level of import was observed with 0.5 mM KCl plus CCCP as compared with 4 – 5 mM KCl without CCCP. Notably, Nicholls (1974, 1982) has shown that addition of a protonophore to mitochondria having a valinomycin-induced potassium diffusion potential at an external KCl concentration of 0.5 mM and a blocked electron transport chain leads to a moderate reduction of the membrane potential to the level which is obtained in the absence of protonophores at a KCl concentration of ~4 mM. With protonophores present, the matrix becomes acidic as the ΔpH is inverted and the total protonmotive force is completely and immediately broken down.

Thus, the import of ADP/ATP carrier appears to be dependent on the size of the membrane potential and independent of the size of the total protonmotive force.

Earlier observations on the inhibition of import of free precursor proteins by valinomycin plus high K^+ and lack of inhibition by nigericin (Schleyer *et al.*, 1982) are in agreement with our conclusions. However, these data alone were not sufficient to show that $\Delta\psi$ and not Δp is the driving force for import. (i) Valinomycin plus K^+ dissipates $\Delta\psi$, but not ΔpH, and inhibits the import of, e.g., ADP/ATP carrier; however, it cannot be excluded that the remaining ΔpH in this case is too low to fulfill the energy requirement (Randall, 1985). Furthermore, a possible K^+/H^+ exchange could reduce the remaining ΔpH (Azzone *et al.*, 1978). (ii) Nigericin does not inhibit the import of mitochondrial ADP/ATP carrier protein; this is also the case when the external potassium concentration is low (N.Pfanner, unpublished). In this situation ΔpH but not $\Delta\psi$ is diminished (Nicholls, 1982); yet it is possible that the pre-existing ΔpH is too low to reveal a significant effect on import.

Discussion

Import of ADP/ATP carrier from the receptor-bound state can be performed in a simple buffer system with the same efficiency as in reticulocyte lysate. This import is very rapid compared with the binding of the precursor to mitochondrial receptors. Using this buffer system we can show that the import of a mitochondrial precursor protein can be driven by a valinomycin-mediated potassium diffusion potential. Creation of a ΔpH does not affect the import and the protonophore CCCP (which completely inhibits the import driven by a proton potential) cannot abolish the import driven by a potassium diffusion potential. We suggest that the membrane potential itself, but not the total protonmotive force, is the driving force for import.

These results clearly argue against a mechanism of protein import in which the movement of precursors is triggered or accompanied by a movement of protons from the outside to the inside driven by the total protonmotive force. Thus precursor import differs from most other processes depending on an energized mitochondrial inner membrane.

Amino-terminal peptide extensions of mitochondrial precursor proteins carry abundant positive charges and very few, if any, negative charges (Kaput *et al.*, 1982; Maarse *et al.*, 1984; Marres *et al.*, 1985; Sadler *et al.*, 1984; Takiguchi *et al.*, 1984; Viebrock *et al.*, 1982; Wright *et al.*, 1984). The ADP/ATP carrier which is synthesized without a peptide extension possesses a large number of positively charged groups, and also clusters of positive charges (Aquila *et al.*, 1982; Arends and Sebald, 1984). The data presented here would agree with an electrophoretic effect of the membrane potential on positively charged domains of precursor proteins. Independent evidence suggests that the transfer of a small amino-terminal piece of the precursors to ATPase β-subunit and to cytochrome c_1 requires an energized inner membrane, but that the translocation of the remaining major part of the protein does not (Schleyer and Neupert, in preparation).

At present, it cannot be determined whether an electrophoretic effect on the additional sequences is indeed the function of the membrane potential or not. There are other possible roles or additional roles, such as an influence on the bilayer structure of the membrane leading to a destabilisation of the arrangement of phospholipid molecules so that hydrophilic domains of the precursors can be guided through the membranes. Furthermore, the membrane potential could trigger a conformational or topological

change of the receptor-bound precursors to guide them into a translocation-competent position. It is also possible that the membrane potential might play a role in the formation or maintenance of translocation contact sites between the outer and inner mitochondrial membranes.

Employing the buffer system described here, it is possible in principle to measure the absolute magnitude of $\Delta\psi$ required for import. A rough calculation of the size of the diffusion potential generated in the experiments can be made on the assumption of a matrix concentration of K^+ of 40 – 120 mM; such values have been reported for mitochondria from various sources (Scarpa, 1979; Nicholls, 1982). A potential of 20 – 40 mV is then able to drive import of the ADP/ATP carrier. However, it will be extremely difficult, if not impossible, to measure reliably these and even lower potentials to answer the interesting question as to whether a threshold potential is required which would, for example, point to a gating effect.

The export of proteins in bacteria and the import of proteins into chloroplasts show similarities to the import of proteins into mitochondria in a number of respects. In particular, the existence of amino-terminal peptide extensions, the requirement of energy for transmembrane movement or membrane insertion and a post-translational translocation have been described (Date *et al.*, 1980a, 1980b; Grossman *et al.*, 1980; Müller and Blobel, 1984; Randall, 1985; Randall and Hardy, 1984; Zimmermann and Wickner, 1983). However, it appears possible that the forms of energy are different for these three transport processes. (i) For chloroplasts a requirement of ATP has been described both for the transport across the envelope membranes and across the thylakoid membranes (Grossman *et al.*, 1980). (ii) The export of proteins in bacteria needs an energized plasma membrane (not ATP) (for review, see Randall, 1985). However, the membrane potential shows the opposite orientation with respect to the direction of protein transport as compared with mitochondria; all amino-terminal peptide extensions present on secreted proteins which were studied until now have a net positive charge (von Heijne, 1984). Daniels *et al.* (1981) proposed a model where the membrane potential itself (not the total protonmotive force) is required; the amino-terminal sequences of the precursor proteins would form a hairpin structure which could be aligned as a net dipole in response to the electrical field. On the other hand, Bakker and Randall (1984) reported that total protonmotive force is required for the export of β-lactamase in *Escherichia coli*. They proposed a different model, implying either an antiport of protons, a symport of hydroxyl anions or a conformational change of membrane structures.

The possible requirement of different forms of energy in bacteria, chloroplasts and mitochondria need not be a contradiction. One can imagine that the required energy sources were adapted to the particular energetics of the various membrane systems.

Materials and methods

Growth of Neurospora cells and preparation of mitochondria

N.crassa (wild-type 74 A) was grown as decribed (Schleyer *et al.*, 1982). Hyphae were harvested by filtration. 1 g of hyphae was ground with 1.5 g quartz sand and 0.5 ml SEM medium (0.25 M sucrose, 1 mM EDTA and 10 mM MOPS, adjusted to pH 7.2 with KOH or NaOH) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), in a mortar for 3 min. After addition of 1.5 ml SEM medium (containing PMSF) mitochondria were re-isolated by differential centrifugation (Sebald *et al.*, 1979). Mitochondria were washed once in SEM medium without PMSF and suspended at a proton concentration of 5 mg/ml in SEM (without PMSF).

Binding and import of ADP/ATP carrier synthesized in reticulocyte lysates

Precursor to ADP/ATP carrier was synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine (specific radioactivity 500 Ci/mmol, Amersham Buchler) as previously described (Schleyer *et al.*, 1982). To produce 'no synthesis lysates' lacking mitochondrial precursor proteins, [^{35}S]methionine and *N. crassa* RNA were omitted, and lysates were not incubated for nuclease treatment and protein synthesis. Post-ribosomal supernatants were prepared and supplemented as described (Schleyer *et al.*, 1982). *In vitro*-synthesized ADP/ATP carrier was bound to, or imported into, mitochondria by incubating labelled reticulocyte lysate with isolated *Neurospora* mitochondria at 25°C. Times of incubation and further additions are given in the figure legends. Where indicated a combination of antimycin A and oligomycin (4 μM and 10 μM final concentrations, added from a 100-fold concentrated stock solution in ethanol) or a combination of valinomycin, antimycin A and oligomycin (0.1 μM, 4 μM and 10 μM final concentrations, respectively, added from a 100-fold concentrated stock solution in ethanol) was included. CCCP was added from a 200-fold concentrated stock solution in ethanol; in this case the combination of valinomycin, antimycin A and oligomycin was also added from a 200-fold concentrated stock solution in ethanol. Potassium or sodium ascorbate and TMPD (4 mM and 0.2 mM final concentrations) and potassium cyanide (1 mM final concentration) were added from 50-fold concentrated solutions in water, respectively. All reactions were made chemically identical by adding the same volume of inhibitor-free solutions to the respective control samples.

Samples were then incubated for 15 min at 0°C and SEM or proteinase K in SEM (20 μg/ml final concentration) were added. After 30 min at 4°C PMSF (1 mM final concentration) was added to all samples and incubation was continued for another 5 min at 4°C. Where indicated mitochondria were re-isolated prior to protease treatment and a second incubation at 25°C was performed.

Immunoprecipitation and electrophoretic analysis

The samples were transferred into new Eppendorf cups, and mitochondria were re-isolated by centrifugation for 12 min at 17 300 *g*. The tubes containing the mitochondrial pellets were re-centrifuged for 20 s in an Eppendorf centrifuge to pellet residual fluid from the tube wall. Mitochondria were solubilized with 400 μl of 1% (w/v) Triton X-100, 0.3 M NaCl, 5 mM EDTA, 10 mM Tris, adjusted to pH 7.5 with HCl, in the presence of 0.1 mM PMSF. The mixtures were freed from insoluble material by centrifugation for 15 min at 27 000 *g*. Sufficient amounts of antiserum against ADP/ATP carrier and Protein-A-Sepharose (Pharmacia) were added. The time for interaction of antibody with antigen, and for the binding of immune complexes to Protein-A-Sepharose was 60 min. The beads were then washed and dissociated as described (Schleyer *et al.*, 1982). SDS-polyacrylamide gel electrophoresis (Schleyer *et al.*, 1982) and fluorography (Chamberlain, 1979) were carried out as described. Quantitation of the immunoprecipitated ADP/ATP carrier was performed by densitometry of the films (Kodak X-Omat AR) at 436 nm. Multiple exposures of each film were examined to assure that the exposure analysed was in the linear response range of the film. Results are expressed in arbitrary units such that the respective maximal value of ADP/ATP carrier in each experiment equals 100.

Miscellaneous

Mitochondrial protein was determined using the method of Bradford (1976).

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