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Requirement of a Membrane Potential for the Posttranslational Transfer of Proteins into Mitochondria

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Posttranslational transfer of most precursor proteins into mitochondria is dependent on energization of the mitochondria. Experiments were carried out to determine whether the membrane potential or the intramitochondrial ATP is the immediate energy source. Transfer *in vitro* of precursors to the ADP/ATP carrier and to ATPase subunit 9 into isolated *Neurospora* mitochondria was investigated. Under conditions where the level of intramitochondrial ATP was high and the membrane potential was dissipated, import and processing of these precursor proteins did not take place. On the other hand, precursors were taken up and processed when the intramitochondrial ATP level was low, but the membrane potential was not dissipated. We conclude that a membrane potential is involved in the import of those mitochondrial precursor proteins which require energy for intracellular translocation.

The great majority of mitochondrial proteins are coded for by nuclear genes. They are synthesized on cytoplasmic ribosomes as precursor proteins, most but not all of which have higher apparent molecular weights than the mature proteins. These precursor proteins are imported into the mitochondria in a posttranslational event [1–11].

The posttranslational transfer *in vivo* and *in vitro* of most, though not all, mitochondrial proteins has been shown to be dependent on the energization of the mitochondria [12–15]. In this study we have investigated which form of energy is required for the transfer of proteins into the inner mitochondrial membrane. We have employed various uncouplers, ionophores, and inhibitors of oxidative phosphorylation, ADP/ATP translocation and electron transport to decide whether the direct force for import is the membrane potential or the intramitochondrial ATP. Two proteins of the inner mitochondrial membrane were studied: the ADP/ATP carrier, which is not proteolytically processed during transfer into mitochondria [4], and the dicyclohexylcarbodiimide-binding subunit of oligomycin-sensitive ATPase (subunit 9 of 'proteolipid'), which undergoes proteolytic cleavage [14, 16].

All results obtained are compatible with the view that it is the membrane potential which is required for protein transfer, and that ATP in the mitochondrial matrix is not the direct energy source.

MATERIALS AND METHODS

Growth of Neurospora *Cells and Preparation of Mitochondria*

Neurospora crassa (wild type 74 A) was cultivated in Vogel's minimal medium [17] supplemented with 2% sucrose. The inoculum was 10^9 conidia/l. After growth at 25 °C under vigorous aeration for 16 h, hyphae were harvested by filtration (about 4 g wet weight/l). They were converted to spheroplasts as described [5]. Mitochondria were isolated after breaking the spheroplasts in a Dounce homogenizer [5].

Rat liver mitochondria were isolated in a medium containing 250 mM sucrose, 2 mM EDTA, 20 mM Tris/HCl, pH 7.3, after homogenization of rat liver in a glass/Teflon homogenizer. The 10-min $750 \times g$ supernatant was centrifuged for 10 min at $7700 \times g$. The resulting crude mitochondrial pellet was washed twice in isolation medium by resuspending and pelleting at $4300 \times g$ for 10 min. Mitochondria were resuspended in isolation medium at a protein concentration of about 30 mg/ml and kept on ice.

Synthesis of Neurospora *Proteins in Reticulocyte Lysates*

Reticulocyte lysates were prepared according to Hunt and Jackson [18], except that rabbits were bled on the seventh day after beginning the injections of phenylhydrazine. The lysates were stored in aliquots of 0.8 ml at −75 °C. Protein synthesis was carried out after nuclease treatment according to Pelham and Jackson [19]. Nucleic acids from *N. crassa* were isolated by phenol extraction of frozen hyphae as described [4]. 0.5 mCi [^{35}S]methionine (≈ 1000 Ci/mmol, NEN Chemicals, Boston, MA) were used per 1.0 ml lysate. After 60 min of protein synthesis *in vitro*, a postribosomal supernatant was prepared by centrifugation for 1 h at $166000 \times g$ and unlabelled methionine and sucrose were added to final concentrations of 0.25 mM and 0.3 M, respectively.

Transfer of Precursor Proteins from the Reticulocyte Lysate into Mitochondria

Mitochondria, prepared from *Neurospora* spheroplasts, were resuspended in 0.3 M sucrose, 1 mM EDTA, 30 mM Tris/HCl, pH 7.6, and gently homogenized in a glass/Teflon

Abbreviations. $CF_3OPhzC(CN)_2$, carbonylcyanide trifluoromethoxyphenylhydrazone; $ClPhzC(CN)_2$, carbonylcyanide *m*-chlorophenylhydrazone; $PhMeSO_2F$, phenylmethanesulfonyl fluoride.

Enzymes. Proteinase K (EC 3.4.21.14); oligomycin-sensitive ATPase (EC 3.6.1.3).

homogenizer. To 20 µl of this suspension containing 0.20–0.22 mg protein, 180 µl postribosomal supernatant of a reticulocyte lysate was added. Inhibitors were given by adding 2 µl or 4 µl from stock solutions in ethanol or acetone. The control incubation received an equal volume of ethanol or acetone. All of these procedures were carried out at 0 °C. The suspensions were then incubated at 25 °C for 60 min. After cooling to 0 °C, mitochondria were pelleted from one half by centrifugation for 12 min at 17300 × *g*. Immunoprecipitation was carried out as described below. The other half was incubated for 60 min at 4 °C after adding carboxyatractyloside and proteinase K to final concentrations of 1.2 µM and 0.1 mg/ml, respectively. Protease treatment was stopped by adding 12 µl 0.1 M $PhMeSO_2F$ in ethanol. The samples were centrifuged for 12 min at 17300 × *g*. The mitochondrial pellets were analysed for transferred proteins as described below.

For transfer of subunit 9, mitochondria were directly resuspended in a postribosomal supernatant of a reticulocyte lysate which contained inhibitors when indicated. Mitochondria were re-isolated by centrifugation and analysed as described below.

Immunoprecipitation of ADP/ATP Carrier

When the ADP/ATP carrier was to be immunoprecipitated, mitochondrial pellets were resuspended in 1.0 ml of 0.3 M KCl, 10 mM Tris/HCl, pH 7.5; to this was added 100 µl of 10 mM *p*-chloromercuribenzoate. Mitochondria were lysed by addition of 55 µl 20% Triton X-100 in 10 mM Tris/HCl, pH 7.5, and the lysates centrifuged for 15 min at 20000 × *g*. After the supernatants were collected, rabbit antiserum directed against ADP/ATP carrier [5] (1/10 the amount required for direct immunoprecipitation) was added, and the mixture incubated overnight at 4 °C. An amount equal to twice the volume of antiserum was then added of a 10% (w/v) suspension of *Staphylococcus aureus* cells, prepared according to Kessler [20]. Samples were shaken for 30 min at 4 °C. After pelleting in an Eppendorf centrifuge for 2 min, cells were washed twice with 1% Triton X-100, 0.9% NaCl, 10 mM Tris/HCl, pH 7.5, and twice with the same solution without Triton. The final pellets were taken up in 50 µl 2% (w/v) sodium dodecylsulfate, 5% (v/v) 2-mercaptoethanol, 10% glycerol, 60 mM Tris/HCl, pH 6.8, and kept at 95 °C for 5 min. After the *S. aureus* cells had been spun down, the supernatant was analysed by gel electrophoresis in the presence of dodecylsulfate according to Laemmli [21] using 17.5% acrylamide. Gels were stained with Coomassie brilliant blue, dried and autoradiographed. When indicated, the stained gels were treated with sodium salicylate for fluorography [22].

Immunoprecipitation of Subunit 9 of Mitochondrial ATPase

Subunit 9 was isolated from *Neurospora* mitochondria according to Sebald et al. [23]. For preparation of antibodies, 0.5 mg of the protein in 0.75 ml water was mixed with an equal volume of complete Freund's adjuvant (Behringwerke, Marburg) and injected subcutaneously into the neck region of a rabbit. This procedure was repeated four times at weekly intervals. After another week, blood was drawn from the ear vein. Immunoprecipitation was carried out under the following conditions: mitochondria (0.5–1.0 mg protein) were lysed for 10 min in 1 ml of 1% Triton X-100, 0.3 M KCl, 1 mM $PhMeSO_2F$, 10 mM Tris/HCl, pH 7.5, and freed from insoluble material by centrifugation for 15 min at 20000 × *g*. 0.5 ml of antiserum was added to the supernatant and the mixture kept at 4 °C for 16 h. The precipitate was collected and washed as described [5]. The immunoprecipitates were dissociated in 60 µl of 2% sodium dodecylsulfate, 5% (v/v) 2-mercaptoethanol, 60 mM Tris/HCl, pH 6.8, for 1 h at 4 °C and then heated to 95 °C for 2 min. Electrophoresis was performed in gels containing 15% acrylamide [21].

For immunoprecipitation of subunit 9 after transfer into rat liver mitochondria, mitochondria were lysed and freed from insoluble material in the same way as *Neurospora* mitochondria. An appropriate amount of antiserum was adsorbed to protein-A–Sepharose (Pharmacia, Uppsala). The protein-A–Sepharose–IgG complex was washed twice with 1% Triton X-100, 0.3 M KCl, 10 mM Tris/HCl, pH 7.5, and added to the mitochondrial lysate. Samples were shaken for 20 min at 4 °C. The Sepharose beads were washed as described [5]. Dissociation was carried out as mentioned above.

Determination of ATPase Activity in Whole Mitochondria

Mitochondria were isolated from *Neurospora* cells after grinding with sand in a mortar [24]. The isolation medium contained 0.25 M sucrose, 1 mM EDTA, 10 mM Tris/acetate, pH 7.5. The mitochondrial pellets were resuspended in isolation medium plus 5 mM ATP at a protein concentration of 5–10 mg/ml. The suspensions were kept on ice.

The hydrolysis of ATP was assayed by measuring the amount of inorganic phosphate released, as described by Tzagaloff [25], except that the assay buffer additionally contained 0.25 M sucrose. The amount of mitochondrial protein added to the reaction mixture varied over 20–30 µg.

Protein Synthesis in Isolated Mitochondria

Incorporation of [^{3}H]leucine into isolated mitochondria was measured as described earlier [26]. Briefly, mitochondria were isolated from cells by grinding with sand and resuspended in the incubation medium as described [26].

RESULTS

Transfer of ADP/ATP Carrier and ATPase Subunit 9 into Mitochondria Is Inhibited by Protonophores and Valinomycin

Protonophores and the ionophore valinomycin inhibit the transfer into isolated mitochondria of ADP/ATP carrier synthesized *in vitro*, but not its binding to mitochondria. At 4 µM added $ClPhzC(CN)_2$ the transfer of carrier was inhibited by 50% (Fig. 1A). This concentration of $ClPhzC(CN)_2$ is high compared to that necessary to uncouple ATP synthesis from respiration in isolated mitochondria [27]. The high concentration required may be the result of unspecific binding of $ClPhzC(CN)_2$ to the large amount of protein, especially hemoglobin, in the reticulocyte lysate, thus reducing the effective concentration of uncoupler. Furthermore, cysteine, which is known to inactivate $ClPhzC(CN)_2$ [27], was present in the lysate. $CF_3OPhzC(CN)_2$ blocked transfer of ADP/ATP carrier at a concentration of about 2 µM (Fig. 1B). Dinitrophenol was able to inhibit transfer at a concentration of 0.3 mM almost completely (Fig. 1C).

The ionophore valinomycin also blocked transfer of the newly synthesized ADP/ATP carrier. Valinomycin was effective at a concentration as low as 0.07 µM (about 70 pmol/mg

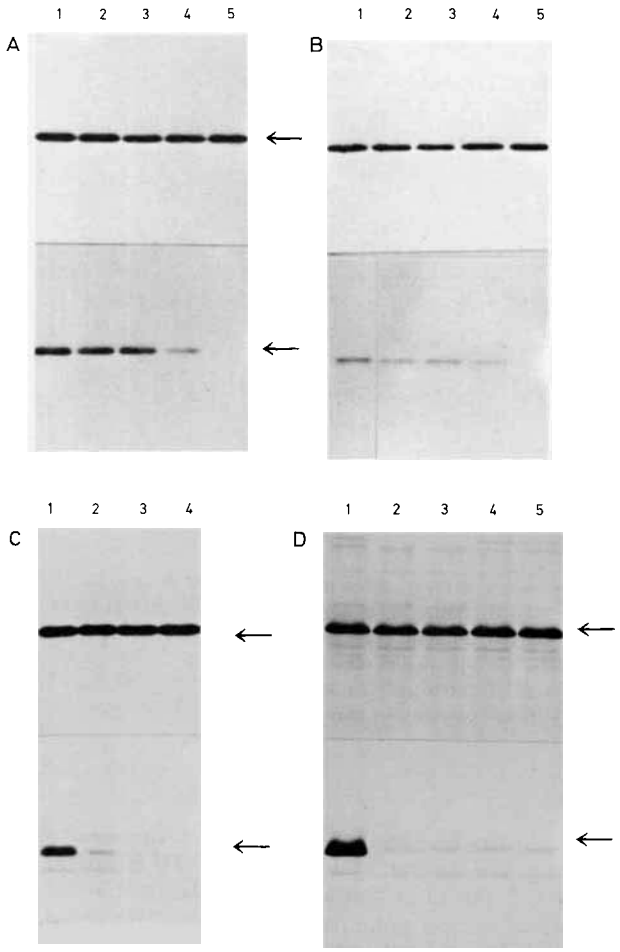

Fig. 1. *Uncouplers and valinomycin inhibit transfer of ADP/ATP carrier into mitochondria in a reconstituted system.* The supernatant of a reticulocyte lysate was prepared after synthesis of [^{35}S]methionine-labelled proteins directed by *Neurospora* RNA. Mitochondria were isolated from *Neurospora* hyphae after conversion to spheroplasts and resuspended in the supernatant. Samples were incubated for 60 min at 25 °C in the absence and presence of (A) $ClPhzC(CN)_2$, (B) $CF_3OPhzC(CN)_2$, (C) dinitrophenol or (D) valinomycin at the indicated concentrations. One half of each sample was then centrifuged to obtain mitochondria. The other half was incubated with 0.1 mg/ml proteinase K for 60 min; then $PhMeSO_2F$ was added. Then mitochondria were isolated from these samples also. After lysis with Triton X-100, ADP/ATP carrier was immunoprecipitated. The immunoprecipitates were analysed by gel electrophoresis in the presence of dodecylsulfate and the gels were fluorographed. Upper gels: without proteinase K treatment; lower gels: with proteinase K treatment. (A) $ClPhzC(CN)_2$ concn: (1) 0; (2) 1.6 μM; (3) 3.1 μM; (4) 6.2 μM; (5) 12.5 μM. (B) $CF_3OPhzC(CN)_2$ concn: (1) 0; (2) 0.25 μM; (3) 1.0 μM; (4) 4.1 μM; (5) 12.5 μM. (C) Dinitrophenol concn: (1) 0; (2) 0.1 mM; (3) 0.3 mM; (4) 1.0 mM. (D) Valinomycin concn: (1) 0; (2) 0.08 μM; (3) 0.2 μM; (4) 0.7 μM; (5) 2.0 μM

mitochondrial protein) (Fig. 1D). Since the K^+ concentration in the reconstituted medium is high (about 90 mM) [28], breakdown of the membrane potential must occur which is accompanied by a non-stoichiometric extrusion of H^+ [29]. The newly synthesized carrier protein which was transferred into mitochondria was resistent to proteinase K concentrations up to 0.4 mg/ml in the same fashion as the assembled mature ADP/ATP carrier. In contrast, the precursor bound to mitochondria in the presence of valinomycin was completely digested by concentrations of proteinase K as low as 0.010 mg/ml.

Table 1. *Effects of various uncouplers and inhibitors on the ATPase activity of whole* Neurospora *mitochondria*
The values measured (11 determinations) gave an average of 2.15 μmol ATP hydrolysed min^{-1} (mg mitochondrial protein)$^{-1}$. The control values were set to 100 in each individual experiment. Concentrations of inhibitors were: oligomycin 5 μM; $ClPhzC(CN)_2$ 12.5 μM; valinomycin/K^+ 2 μM/60 mM

System	ATPase activity
	% control
Control	100
+ oligomycin	56 ± 16
+ $ClPhzC(CN)_2$	156 ± 25
+ oligomycin + $ClPhz(CN)_2$	48 ± 18
+ valinomycin/K^+	157 ± 35
+ oligomycin + valinomycin/K^+	47 ± 26

The effects of $ClPhzC(CN)_2$ and valinomycin on the transfer of the precursor *in vitro* to ATPase subunit 9 which is proteolytically processed upon transfer into mitochondria [14, 23] were also investigated. Processing and transfer of this precursor was inhibited by both compounds. Binding of the precursor to putative receptor sites on the mitochondria was not affected (see also [14]). $ClPhzC(CN)_2$ and valinomycin have also been found to block the transfer and processing of subunits of the cytochrome bc_1 complex (core protein I, cytochrome c_1, and Fe/S protein) (M. Teintze, H. Weiß and W. Neupert, unpublished).

The ionophore nigericin exchanges K^+ versus H^+ in a stoichiometric way, thus leading to a breakdown of the proton gradient without affecting the membrane potential [30, 31]. Nigericin at a concentration of 2 μM did not inhibit transfer of ADP/ATP carrier and the transfer and processing of ATPase subunit 9 was not blocked. Apparently, a membrane potential but not a proton gradient is necessary to drive uptake of precursor proteins.

$ClPhzC(CN)_2$ and Valinomycin Stimulate an Oligomycin-Sensitive ATPase Activity in Isolated Neurospora *Mitochondria*

Inhibition of transfer by $ClPhzC(CN)_2$ and valinomycin could be caused either by the breakdown of the membrane potential directly or by a secondary phenomenon. One major consequence of the dissipation of the membrane potential could be a large decrease of the level of intramitochondrial ATP, due to an increased activity of the oligomycin-sensitive ATPase [32].

The ATPase activity of isolated *Neurospora* mitochondria was measured in the absence and presence of various uncouplers and inhibitors (Table 1). The isolated mitochondria showed an ATPase activity, in agreement with the observation by various authors that isolated mitochondria from fungi are often loosely coupled [33, 34]. $ClPhzC(CN)_2$ as well as valinomycin/K^+ stimulated ATP hydrolysis in whole *Neurospora* mitochondria. This is explained by the tendency of the ATPase to maintain a membrane potential by pumping out protons to compensate for the influx of H^+ or K^+ [29]. This ATPase activity was strongly inhibited by oligomycin, to a level much below that of control mitochondria. Thus oligo-

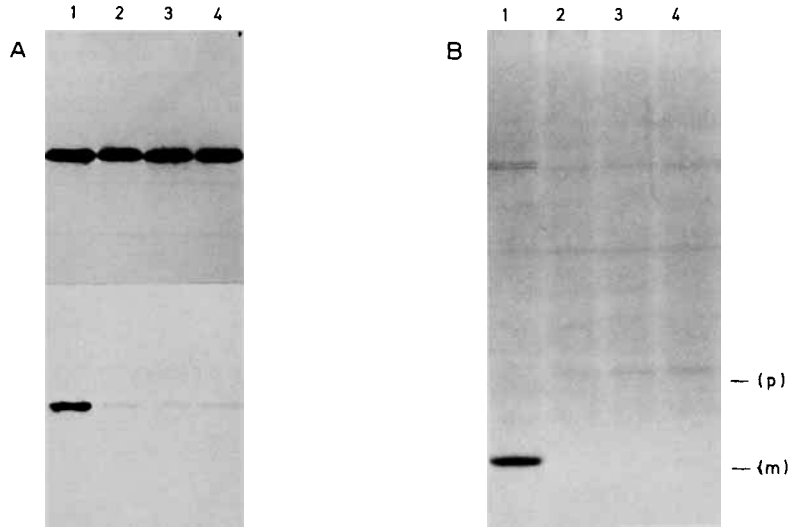

Fig. 2. *High intramitochondrial ATP concentration does not promote transfer of precursor proteins when mitochondria are uncoupled.* Transfer of precursor proteins *in vitro* was carried out in the presence or absence of $ClPhzC(CN)_2$, valinomycin, oligomycin, and ATP. (A) Transfer of ADP/ATP carrier. Upper gels: without proteinase K treatment; lower gels: with proteinase K treatment. (1) Control; (2) 1 μM valinomycin plus 10 μM oligomycin; (3) 12.5 μM $ClPhzC(CN)_2$ plus 10 μM oligomycin; (4) 12.5 μM $ClPhzC(CN)_2$ plus 10 μM oligomycin plus 2 mM ATP; arrow indicates the position of the mature ADP/ATP carrier. (B) Transfer of ATPase subunit 9. (1) Control; (2) 1 μM valinomycin plus 10 μM oligomycin; (3) 12.5 μM $ClPhzC(CN)_2$ plus 10 μM oligomycin; (4) 12.5 μM $ClPhzC(CN)_2$ plus 10 μM oligomycin plus 2 mM ATP; (p) precursor; (m) mature subunit 9

mycin can be employed to determine whether the inhibition of transfer by $ClPhzC(CN)_2$ and valinomycin is due to the ATPase-stimulating activity of these compounds.

Oligomycin and Carboxyatractyloside Do Not Inhibit Transfer of Precursor Proteins

Neither oligomycin nor carboxyatractyloside nor a combination of both inhibited the transfer of newly synthesized ADP/ATP carrier into a protease-resistant location. Additionally, when mitochondria were preincubated for 10 min at 25 °C with oligomycin and carboxyatracyloside to deplete the intramitochondrial ATP pool, the mitochondria still imported precursor proteins. These data suggest that ATP is not required for import of the precursor proteins, because in the presence of both inhibitors ATP can neither be synthesized via oxidative phosphorylation nor imported via the ADP/ATP carrier.

Oligomycin should relieve the inhibition exerted by $ClPhzC(CN)_2$ and valinomycin, if ATP were the immediate energy source, because it blocks the ATPase induced by these compounds (see Table 1). However when oligomycin was added to reactions along with these agents, import and processing remained blocked (Fig. 2A). In a further experiment, mitochondria were first incubated for 5 min with oligomycin before valinomycin was added, to be sure that the ATPase was actually blocked before the onset of the uncoupling effect. Transfer of the ADP/ATP carrier into a protease-resistant location occurred only during the 5-min preincubation with oligomycin.

Conditions have been described under which the mitochondrial ATP level is elevated, but the membrane potential is dissipated. This is achieved by adding oligomycin, $ClPhzC(CN)_2$ and ATP to isolated mitochondria. In the presence of these inhibitors external ATP is readily transported into the matrix space by the ADP/ATP carrier, as has been demonstrated by Heldt et al. [35]. Since the ADP/ATP exchange is electrogenic, the export of ATP is favoured by a membrane potential that is positive outside [36, 37]. In the absence of such a potential, the external and internal ATP pools equilibrate. Oligomycin, in turn, prevents the hydrolysis of the ATP that enters the mitochondria under these conditions, and matrix ATP levels rise above those found in the absence of $ClPhzC(CN)_2$ and oligomycin [35]. Fig. 2A shows that a combination of oligomycin, $ClPhzC(CN)_2$ and ATP blocked the import of ADP/ATP carrier. The same held true for the transfer and processing of ATPase subunit 9 (Fig. 2B). These observations strongly suggest that ATP is not the immediate source of energy that drives import of precursor proteins into mitochondria.

The Same Pattern of Response to Inhibitors Is Observed when Neurospora *Precursor Proteins Are Transferred into Rat Liver Mitochondria*

The effect of the inhibitors employed here, including the combination of oligomycin, $ClPhzC(CN)_2$ and ATP, were originally demonstrated with mitochondria from liver of heart from higher animals [29, 35]. In recent experiments we have found that precursors to mitochondrial proteins of *Neurospora* are imported into isolated rat liver mitochondria. The transported proteins were in a protease-resistant location and were processed to the size of the mature proteins (unpublished). Therefore, the effects of the inhibitors used above were also studied in this heterologous system. As before, $ClPhzC(CN)_2$ or valinomycin inhibited transfer of ADP/ATP carrier (Fig. 3A) and transfer and processing of ATPase subunit 9 (Fig. 3B). Oligomycin did not reverse the effect of $ClPhzC(CN)_2$ and valinomycin. Addition of ATP in the presence of oligomycin and $ClPhzC(CN)_2$ did not promote the transfer of precursors into mitochondria. With ATPase subunit 9, and intermediate-sized form was observed upon transfer into rat liver mito-

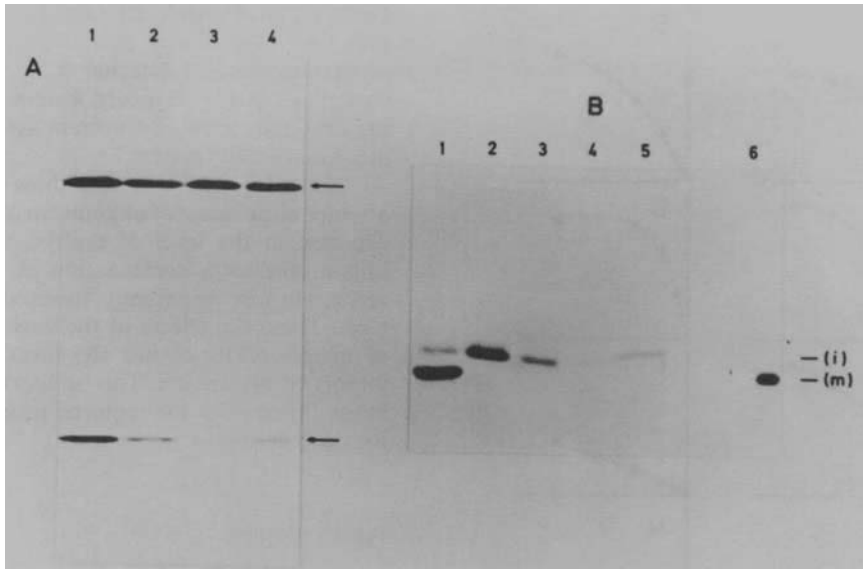

Fig. 3. *Transfer of proteins is also sensitive to uncouplers when* Neurospora *precursors are transferred into rat liver mitochondria.* Transfer *in vitro* was performed as in the previous experiments, with the exception that mitochondria from rat liver were employed in the reconstituted system. (A) Transfer of ADP/ATP carrier. Upper gels: without proteinase K treatment; lower gels: with proteinase K treatment. (1) Control; (2) 12.5 μM $ClPhzC(CN)_2$; (3) 2 μM oligomycin plus 12.5 μM $ClPhzC(CN)_2$; (4) 2 μM oligomycin plus 12.5 μM $ClPhzC(CN)_2$ plus 2 mM ATP. (B) Transfer of ATPase subunit 9. (1) Control; (2) 1 μM valinomycin; (3) 12.5 μM $ClPhzC(CN)_2$; (4) 12.5 μM $ClPhzC(CN)_2$ plus 10 μM oligomycin; (5) 12.5 μM $ClPhzC(CN)_2$ plus 10 μM oligomycin plus 1 mM ATP; (6) mature subunit 9, immunoprecipitated from ^{35}S-labelled *Neurospora* mitochondria; (m) mature subunit 9; (i) intermediate form

chondria which was not seen when the precursor was transferred into *Neurospora* mitochondria. This intermediate form was sensitive to added protease. Its significance remains to be determined.

Inhibitors of Respiration Affect Import of Precursor Proteins into Mitochondria

Inhibitors of mitochondrial electron transport acting in the various regions of the respiratory chain were tested for their ability to inhibit import of precursors. In a parallel experiment, the effect of these inhibitors was studied in the presence of oligomycin, to prevent the generation of a membrane potential by the reversed action of ATP synthase.

In the absence of oligomycin, rotenone inhibited partially the transfer of the ADP/ATP carrier and subunit 9 (Fig. 4). Antimycin A alone or in combination with rotenone had a similar inhibitory effect. KCN did not inhibit the transfer. In the presence of oligomycin a different pattern was observed. Together with rotenone, oligomycin did not lead to further inhibition of transfer. However, when antimycin A or KCN were added together with oligomycin, transfer of ADP/ATP carrier and of subunit 9 were completely blocked (Fig. 4). Preincubation of mitochondria with the inhibitors did not lead to different results.

The incomplete inhibition by the respiratory inhibitors may have several reasons. *Neurospora* mitochondria possess an alternative pathway of electron transport that bypasses the cytochrome bc_1 complex and cytochrome oxidase [38, 39]. Also, rotenone may exert incomplete inhibition of NADH dehydrogenase [38]. Furthermore, electrons can enter the respiratory chain at the level of coenzyme Q, thus mitigating the effect of rotenone. The inhibition of the transfer of precursor proteins by a combination of oligomycin and antimycin A or KCN suggests that, in the presence of these respiratory chain inhibitors alone, a membrane potential can

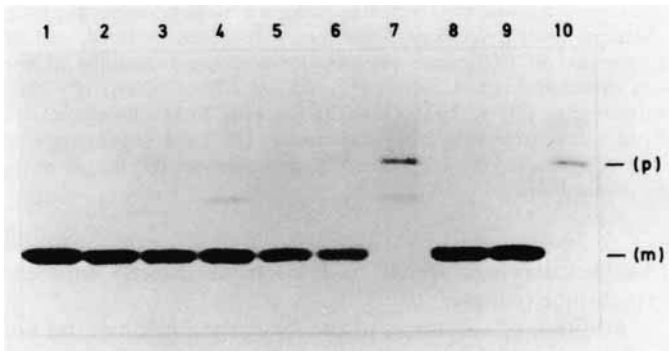

Fig. 4. *Respiratory inhibitors affect transfer and processing of ATPase subunit 9.* Transfer *in vitro* was performed as in Fig. 2, with the following modifications. Spheroplasts were broken and the 5-min 2000 × *g* supernatant was prepared. To this supernatant inhibitors were added when indicated. The mixture was kept at 25 °C for 5 min. Then mitochondria were prepared and resuspended in a postribosomal supernatant containing inhibitors as indicated. (1) control; (2, 3) 10 μM rotenone; (4) 10 μM oligomycin and 10 μM rotenone; (5, 6) 4 μM antimycin A; (7) 10 μM oligomycin and 4 μM antimycin A; (8, 9) 1 mM KCN; (10) 10 μM oligomycin and 1 mM KCN; (1, 2, 5, 8) inhibitors were added to the supernatant of the reticulocyte lysate and then mitochondria were resuspended; (3, 4, 6, 7, 9, 10) inhibitors were added to the 2000 × *g* supernatant and the postribosomal supernatant of the reticulocyte lysate. (p) precursor to subunit 9; (m) mature subunit 9

be generated by the oligomycin-sensitive ATPase. The partial inhibition by oligomycin plus rotenone suggests that a membrane potential is generated by electrons entering the respiratory chain after the NADH dehydrogenase. The complex mixture of the reticulocyte lysate may well contain pertinent substrates. This conclusion is supported by the observation that the inhibition by oligomycin plus antimycin A was relieved by the addition of ascorbate and tetramethyl-

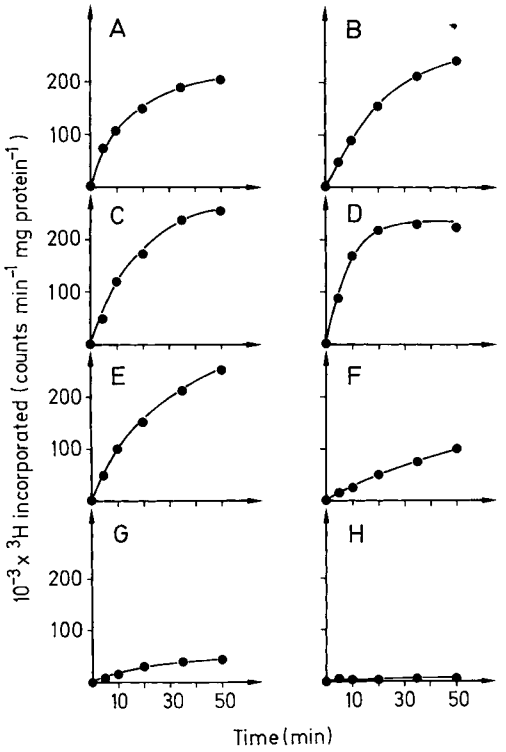

Fig. 5. *Protein synthesis in isolated* Neurospora *mitochondria is dependent on intramitochondrial ATP, but not on the membrane potential.* Isolated *Neurospora* mitochondria were incubated in a medium supporting mitochondrial protein synthesis. Inhibitors were added as listed, and incorporation of [^{3}H]leucine into trichloroacetic-acid-insoluble protein was determined. (A) Control; (B) 12.5 μM $ClPhzC(CN)_2$; (C) 2 μM valinomycin; (D) 12.5 μM $ClPhzC(CN)_2$ plus 2 μM oligomycin; (E) 2 μM valinomycin plus 2 μM oligomycin; (F) 2 μM oligomycin; (G) 2 μM oligomycin plus 1.2 μM carboxyatractyloside; (H) 0.1 mg chloramphenicol/ml

phenylenediamine, which feed electrons directly into the cytochrome oxidase.

Addition of substrates to the incubation medium did not lead to enhanced import of precursor proteins. Apparantly, there are enough substrates to maintain a membrane potential sufficient to drive import.

Protein Synthesis on Isolated Mitochondria as a Measure of the ATP Level in the Matrix

Protein synthesis on mitochondrial ribosomes is dependent on intramitochondrial ATP. Thus the activity of mitochondrial protein synthesis can be employed as a measure of the level of matrix ATP. Protein synthesis was followed in isolated mitochondria which were resuspended in a postribosomal supernatant from *Neurospora*, optimized for incorporation of radioactive amino acids into protein [26] (see Fig. 5). $ClPhzC(CN)_2$ and valinomycin did not inhibit, but rather stimulated incorporation of radioactive amino acids, suggesting that dissipation of the membrane potential leads to enhanced uptake of ATP. Oligomycin alone led to a strong inhibition of protein synthesis, showing that a large part of the matrix ATP comes from respiratory chain phosphorylation when mitochondria are not uncoupled. $ClPhzC(CN)_2$ and valinomycin relieved the inhibition exerted by oligomycin, demonstrating that ATP is efficiently taken up in the absence of a membrane potential but not in its presence. Carboxyatractyloside added together with oligomycin led to a further inhibition of protein synthesis, probably because it blocked import of external ATP. Finally, chloramphenicol completely inhibited incorporation of [^{3}H]leucine, thus showing that mitochondrial protein synthesis was actually being measured in this system.

Taken together, our data show that the uncouplers in the absence or presence of oligomycin did not lead to a measurable decrease in the level of matrix ATP. On the other hand, oligomycin, or a combination of oligomycin and carboxyactractyloside, apparently lowered the mitochondrial ATP level. Thus, the effects of the various inhibitors on the level of matrix ATP are just the reverse of their effects on the import of precursors. This supports the view that the membrane potential is the required form of mitochondrial energy for protein import.

DISCUSSION

All data presented here lead to the conclusion that it is the membrane potential and not the matrix ATP which is required for import of mitochondrial proteins. Membrane potential and matrix ATP are closely interrelated, since according to the chemiosmotic hypothesis a membrane potential can generate ATP from ADP and P_i and, on the other hand, hydrolysis of ATP in the matrix can generate a membrane potential [40]. To discriminate between these two forms of mitochrondrial energy, we employed conditions under which the level of matrix ATP was high and the membrane potential was dissipated. Under these conditions there was no import of precursor proteins. On the other hand, conditions were generated under which matrix ATP was low, but the membrane potential was not destroyed. Under these conditions, import of precursors was not inhibited. The incomplete inhibition by the various respiratory chain blockers would suggest that a relatively low membrane potential is sufficient to support transfer. The inability of nigericin to inhibit import indicates that it is not specifically the H^+ potential, but the membrane potential which is relevant.

In discussing the effects of the various inhibitors employed in this study, the possibility should be considered that in the reconstituted system uncouplers, ionophores, and blockers of phosphorylation and respiration may exert some unspecific effects, and that the mitochondrial membrane potential is not the real target. There are a number of reasons for assuming that this is not the case. First, it is difficult to explain the inhibition by the various substances by a common unspecific effect. Second, transfer of precursors which are not energy-dependent, such as that of cytochrome *c*, is not affected by any of the inhibitors. For cytochrome *c* in particular, we and others have shown that breakage of the outer membrane, which would be a major unspecific effect, impairs uptake of apocytochrome *c* and conversion to holocytochrome *c* [41,42]. Apparently, transfer of cytochrome *c* is a sensitive criterion for integrity of mitochondria. Third, the uncoupler $ClPhzC(CN)_2$ and the ionophore valinomycin did not inhibit but rather stimulated mitochondrial protein synthesis, also a process known to be sensitive to damage of mitochondria. Fourth, no swelling was observed when mitochondria in an ionic medium similar to that of the reconstituted system were treated with valinomycin, arguing against the possibility of rupture of mitochondria. Fifth, energy dependence of protein transfer has also been observed in intact cells [11,12].

The conclusions reached in this study are in conflict with those reached in a study of mitochondrial assembly in *rho*$^-$ mutants of *S. cerevisiae* [13]. These mutants contain mitochondria which are deficient in mitochondrial protein synthesis and thus lack both a functional respiratory chain and a functional oligomycin sensitive ATPase [34]. Thus these cells should be unable to generate a mitochondrial transmembrane potential by respiration or by hydrolysis of ATP, and, indeed, attempts to detect such a potential have been unsuccessful so far [43]. Nevertheless, *rho*$^-$ cells contain mitochondria and, despite of their apparent lack of a membrane potential, they must be capable of importing and processing their cytoplasmically synthesized proteins. It was thus concluded that ATP, which is obtained from the cytoplasm via the ADP/ATP carrier [44], provides the energy for the import [13].

The reason for the different views emerging from our experiments and from the consideration of the situation in *rho*$^-$ yeast cells is not clear. It should, however, be considered that there are ways of generating at least a low mitochondrial membrane potential in *rho*$^-$ cells. One way to do this is by the import of ATP into mitochondria. Since the ADP/ATP translocation is an electrogenic process [36], import of ATP coupled to export of ADP generates a potential, positive on the outside. Actually, *rho*$^-$ mitochondria must import ATP, e.g. to synthesize DNA and RNA [34]. This may generate a membrane potential sufficient to drive import of precursor proteins and transport of ions across the inner mitochondrial membrane.

Interestingly, a requirement for a membrane potential has also been found for the transfer of precursor proteins into or across the cytoplasmic membrane in *Escherichia coli* [45–49].

The uptake of a mature mitochondrial protein, enzymically active aspartate aminotransferase into preparations of rat liver mitochondria has been analysed with respect to its energy requirements. This process has been reported to be inhibited by uncouplers [50]. A recent study employing various inhibitors led the authors to suggest that it depends on a transmembrane pH gradient but not on the membrane potential [51]. This contrasts with our findings and further supports the view that the transfer of precursor proteins into mitochondria is a process different from the transfer of mature assembled enzymes, studied as a model system.

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