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Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria

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We have investigated the energy requirement of mitochondrial protein import with a simplified system containing only isolated yeast mitochondria, energy sources and a purified precursor protein. This precursor was a fusion protein composed of 22 residues of the cytochrome oxidase subunit IV pre-sequence fused to mouse dihydrofolate reductase. Import of this protein required not only an energized inner membrane, but also ATP. ATP could be replaced by GTP, but not by CTP, TTP or non-hydrolyzable ATP analogs. Added ATP did not increase the membrane potential of respiring mitochondria; it supported import even if the proton-translocating mitochondrial ATPase and the entry of ATP into the matrix were blocked. We conclude that ATP exerts its effect on mitochondrial protein import outside the inner membrane.

Key words: mitochondrial protein import/fusion protein/energized inner membrane/ATP effect/potential-sensitive dye

Introduction

Many prokaryotic and eukaryotic proteins must be transported across one or more membranes in order to reach their final destination. What is the energy source required for this transport?

It has recently been found that protein transport across several biological membranes requires ATP. For example, protein translocation across the bacterial plasma membrane requires both ATP and an electrochemical potential across that membrane (Chen and Tai, 1985; Geller *et al.*, 1986). ATP appears to be the only energy source needed to transport proteins into chloroplasts (Grossman *et al.*, 1980; Flügge and Hinz, 1986) or across the endoplasmic reticulum (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Mueckler and Lodish, 1986).

Up to now, mitochondria seemed to be an exception. An initial speculation that protein import into mitochondria required ATP (Nelson and Schatz, 1979) was abandoned when it was shown that import required an energized inner membrane (Gasser *et al.*, 1982; Schleyer *et al.*, 1982). The subsequent demonstration that import could be driven by a K^+-diffusion potential across that membrane (Pfanner and Neupert, 1985) led to the view that protein import into mitochondria required only the electrical component ($\Delta\psi$) of the proton-motive force.

In the past year, progress in the understanding of protein translocation across membranes has suggested that different membrane systems use fundamentally similar transport mechanisms (Schatz, 1986). This prompted us to re-investigate the energy requirement of protein import into mitochondria. Previous studies had been hampered by the fact that protein import into isolated mitochondria could only be measured in the presence of a reticulocyte lysate which served as a source of radiolabeled precursor protein. As such a lysate is generally supplemented with ATP and an ATP-regenerating system to support protein synthesis, and as ATP and other nucleotides can tightly bind to cellular proteins, the presence of a reticulocyte lysate makes it difficult to define the energy source(s) present during *in vitro* import studies.

In the present work, we have replaced the cell-free protein synthesizing system by a highly purified, radiolabeled precursor protein. This protein contains the first 22 residues of the cytochrome oxidase subunit IV pre-sequence fused to the cytosolic 'passenger' protein, mouse dihydrofolate reductase (DHFR). Hurt *et al.* (1984, 1985) have shown that this fusion protein behaves like an authentic mitochondrial precursor protein: it is efficiently imported by energized mitochondria, and cleaved by the matrix-located processing protease, both *in vitro* and *in vivo*. Efficient import and cleavage are still observed if the fusion protein is expressed in *Escherichia coli* in the presence of [^{35}S]sulfate, purified, and added to a buffered suspension of energized yeast mitochondria. Import of this fusion protein, in contrast to that of the purified precursor to the F_1-ATPase beta subunit (Ohta and Schatz, 1984), does not require the presence of cytosolic proteins (Eilers and Schatz, 1986). This simple system allowed us to probe the energy requirement(s) of protein import into mitochondria more reliably than had been possible before. Here we show that protein import into yeast mitochondria requires not only an energized inner membrane, but also ATP outside the inner membrane.

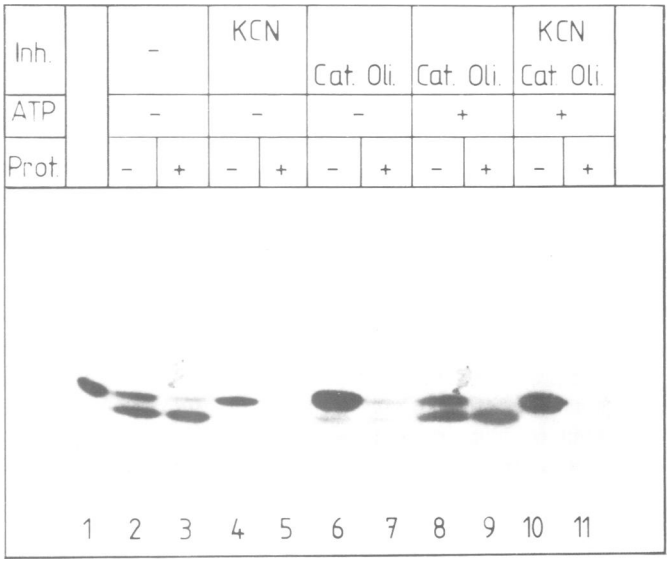

Fig. 1. Import of a purified precursor protein requires both a membrane potential and ATP. Purified, radiolabeled precursor protein (1×10^5 c.p.m., 3×10^8 c.p.m./mg protein) was incubated with isolated yeast mitochondria in the presence of respiratory substrates, succinate and L-malate, and, where indicated, the inhibitors mentioned below. Mitochondria were either left untreated **(lanes 2,4,6,8,10)** or treated with proteinase K (Prot.) afer import **(lanes 3,5,7,9,11)**, to digest precursor bound to the surface (Hurt *et al.*, 1985). They were then re-isolated and analyzed by SDS–gel electrophoresis and fluorography for imported protein. Lane 1 contains a standard corresponding to 10% of the added precursor. Cat., carboxyatractyloside; Oli., oligomycin.

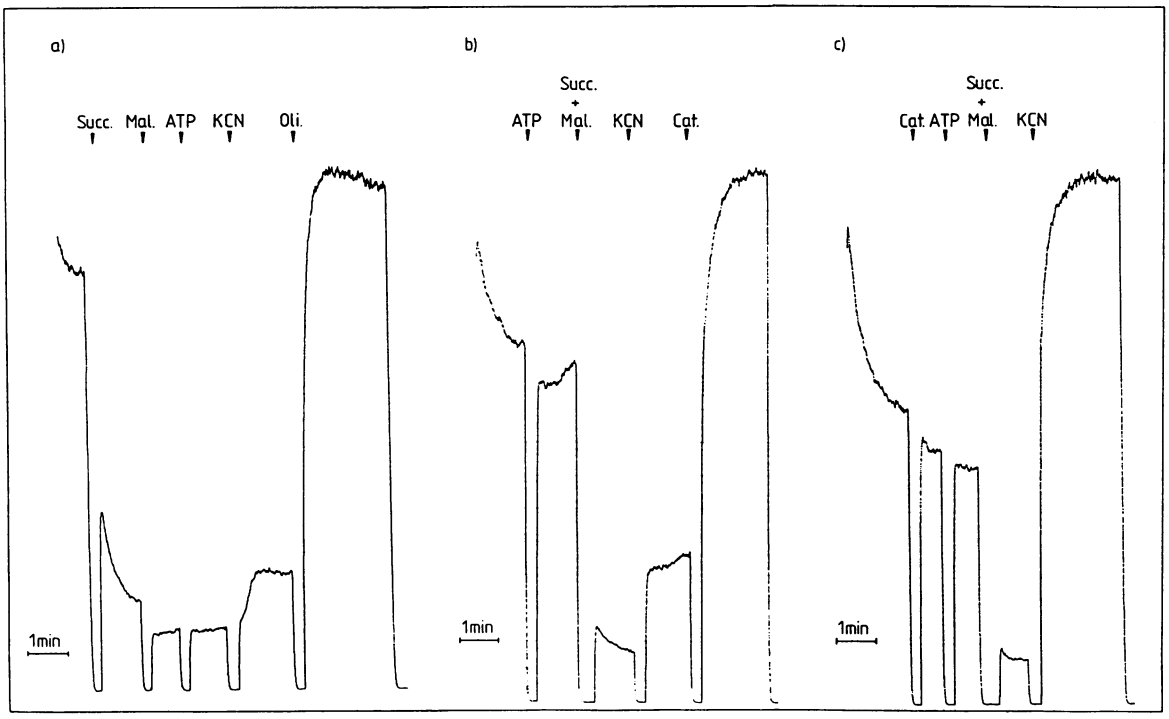

Fig. 2. Potential measurements on isolated mitochondria. Isolated yeast mitochondria were incubated with the potential-sensitive fluorescent dye, diS-C_3-(5) and the fluorescence was recorded. A downward deflection signifies an increase in the membrane potential. Succ., succinate; Mal., L-malate, see also legend to Figure 1.

Results

Both ATP and an energized inner membrane are required for protein import into mitochondria

When the radiolabeled, purified fusion protein (Figure 1, lane 1) was incubated with isolated yeast mitochondria in the presence of the respiratory substrates succinate and malate, it was partly cleaved to the 'mature' form (lane 2) which was inaccessible to externally-added protease (lane 3). This import was energy-dependent: it was blocked by the respiratory inhibitor, cyanide (lanes 4,5). Cyanide prevents not only the generation of a membrane potential, but also respiration-driven ATP synthesis. In order to test whether protein import could be driven by an energized membrane in the absence of ATP, respiration-driven ATP synthesis was blocked by oligomycin (lanes 6–11; for reasons discussed below, these samples also contained carboxyatractyloside, an inhibitor of adenine nucleotide translocation across the mitochondrial inner membrane). Inhibition of ATP synthesis by oligomycin strongly inhibited import of the fusion protein into mitochondria (compare lanes 2 and 6); addition of ATP to the suspending medium restored import (lane 8). Restoration by ATP could not be explained by an effect on the membrane potential since such an effect was prevented by the presence of oligomycin and carboxyatractyloside (see also Figure 2 below). This suggested that import required ATP. However, ATP was not sufficient; an energized inner membrane appeared to be needed as well since inhibition of respiration by cyanide blocked import even in the presence of added ATP (lanes 10,11). As before, the presence of oligomycin and carboxyatractyloside excluded ATP-induced generation of a membrane potential.

In order to corroborate these results, we used a fluorescent cyanine dye to measure the potential across the mitochondrial inner membrane under the conditions described above. In these fluorescence measurements, (Figure 2) a downward deflection signifies an increase of the potential. Figure 2a shows that addition of succinate to isolated mitochondria elicited a near maximal potential which was only slightly enhanced by malate. Addition of ATP gave no further potential increase. This showed directly that the effect of ATP on protein import into respiring mitochondria was not caused by an ATP-induced potential increase. Addition of KCN caused only a modest potential drop

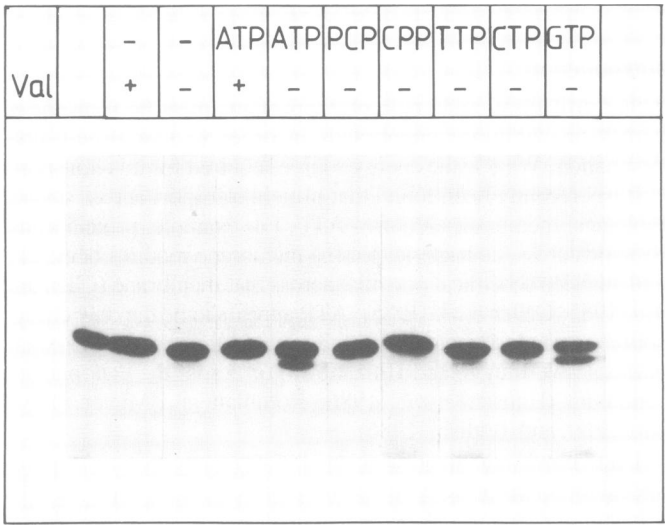

Fig. 3. Nucleotide specificity of protein import into mitochondria. Isolated yeast mitochondria were pre-incubated with glycerokinase (1.4 U/mg mitochondrial protein) and glycerol in the presence of efrapeptin (to deplete endogenous ATP) for 10 min at 10°C. They were then re-isolated and incubated with purified precursor protein in the presence of respiratory substrates and efrapeptin, as described in Figure 1. Where indicated, nucleotides were added at a final concentration of 1 mM. **Lane 1** contains a standard corresponding to 10% of the added precursor protein. VAL, valinomycin, PCP, β, γ-methyleneadenosine-5′-triphosphate, CPP, α, β-methyleneadenosine-5′-triphosphate.

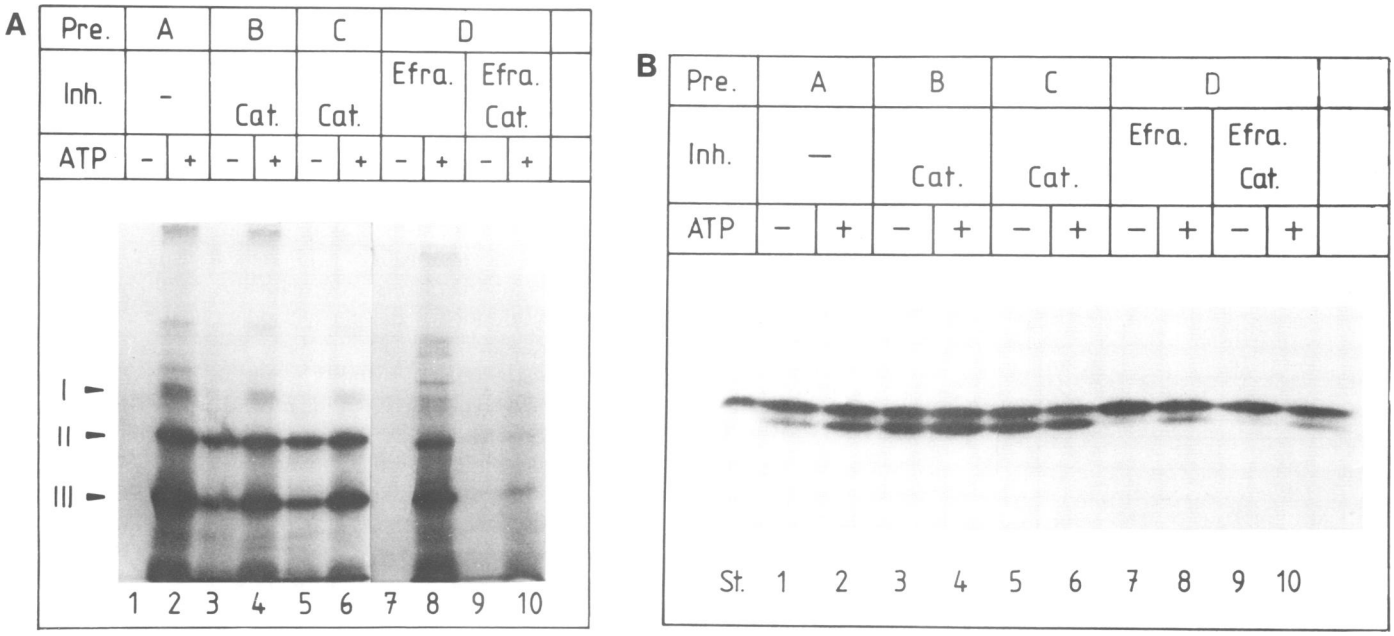

Fig. 4. ATP stimulates mitochondrial protein import on the cytosolic side of the inner membrane. Isolated yeast mitochondria were pre-incubated for 10 min at 10°C in the presence of glucose and respiratory substrates. (A) without further additions, (B) with carboxyatractyloside, (C) with carboxyatractyloside and hexokinase (1 U/mg mitochondrial protein), or **(D)** with efrapeptin and hexokinase. They were then re-isolated and analyzed either for protein synthesis (**panel A**) or for import of precursor (**panel B**) as described in Materials and methods. All other additions are indicated. In panel A, the positions of the three mitochondrially encoded subunits of cytochrome oxidase (I–III) are shown. Efra., efrapeptin; Cat., carboxyatractyloside.

since it prevented only the respiration-induced, but not the ATP-induced potential. The ATP-induced potential could be eliminated by the subsequent addition of oligomycin. The remaining panels of Figure 2 show the following: (i) carboxyatractyloside completely prevented exogenously added ATP from generating a potential (Figure 2b); (ii) ATP in the absence of respiratory substrates induced only a small potential (Figure 2b); (iii) the combination of carboxyatractyloside and KCN completely collapsed the potential even in the presence of respiratory substrates and added ATP (Figure 2c).

We conclude that protein import into mitochondria requires both an energized inner membrane and ATP.

Mitochondrial protein import can be driven by ATP or GTP, but not by CTP, TTP or by non-hydrolyzable ATP analogs

In order to test whether ATP could be replaced by other nucleoside triphosphates or by non-cleavable ATP analogs, yeast mitochondria were pre-incubated with glycerol kinase, Mg^{2+} and glycerol (to deplete any ATP that may have been bound to the mitochondrial surface) and with efrapeptin (a potent inhibitor of the F_1-moiety of mitochondrial ATPase in intact mitochondria; to prevent respiration-driven ATP synthesis). They were then re-isolated by centrifugation, supplemented with respiratory substrates and the various nucleotides listed in Figure 3, and tested for their ability to import the radiolabeled fusion protein. Only ATP and GTP supported significant import; the marginal effects of TTP and CTP probably reflected the fact that these nucleotides could generate ATP from intramitochondrial ADP via mitochondrial nucleoside diphosphokinase. The inactivity of the non-hydrolyzable ATP analogs suggested that ATP and GTP supported protein import via cleavage of the terminal phosphoanhydride bond.

Mitochondrial site of ATP effect

In order to test whether ATP acted inside or outside the mitochondrial inner membrane, we blocked the exchange of adenine nucleotides across the mitochondrial inner membrane with carboxyatractyloside (Klingenberg, 1980). If added ATP supports mitochondrial protein import at a site outside the inner membrane, it should do so even in the presence of carboxyatractyloside. However, this reasoning must be viewed with caution: since the rate of protein import is much smaller than that of oxidative phosphorylation, carboxyatractyloside may be an effective inhibitor of ATP-driven mitochondrial energy coupling reactions, yet allow sufficient leakage of externally added ATP across the inner membrane for slow ATP-driven reactions to occur in the matrix at almost normal rates. A slow, carboxyatractyloside-insensitive exchange of adenine nucleotides across the inner membrane has indeed been found in rat liver mitochondria (Austin and Aprille, 1984).

To control for such a leak, we used intramitochondrial protein synthesis as a measure of the availability of ATP to a very slow ATP-driven reaction inside the inner membrane (Figure 4A). The major products of protein synthesis by isolated yeast mitochondria (cytochrome oxidase subunits I–III) can be readily visualized by SDS–polyacrylamide gel electrophoresis and fluorography (Poyton and Groot, 1975; Ohashi and Schatz, 1980). Freshly isolated yeast mitochondria failed to synthesize proteins even if supplemented with respiratory substrates (lane 1) unless ATP was added as well (lane 2). Addition of carboxyatractyloside allowed continued protein synthesis (lanes 3,4), perhaps by blocking efflux of intramitochondrial ATP or hydrolysis of ATP. Also, it did not eliminate the stimulatory effect of added ATP on mitochondrial protein synthesis (compare lanes 3 and 4 or lanes 5 and 6), suggesting that it did not completely block transport of added ATP to the matrix space. Pre-incubation of mitochondria with glucose and hexokinase (lanes 5,6) had no significant effect (compare lanes 5,6 with lanes 3,4).

Since these results were complicated by the intramitochondrial generation of ATP via oxidative phosphorylation, we blocked the ATP synthase with efrapeptin. Now mitochondrial protein

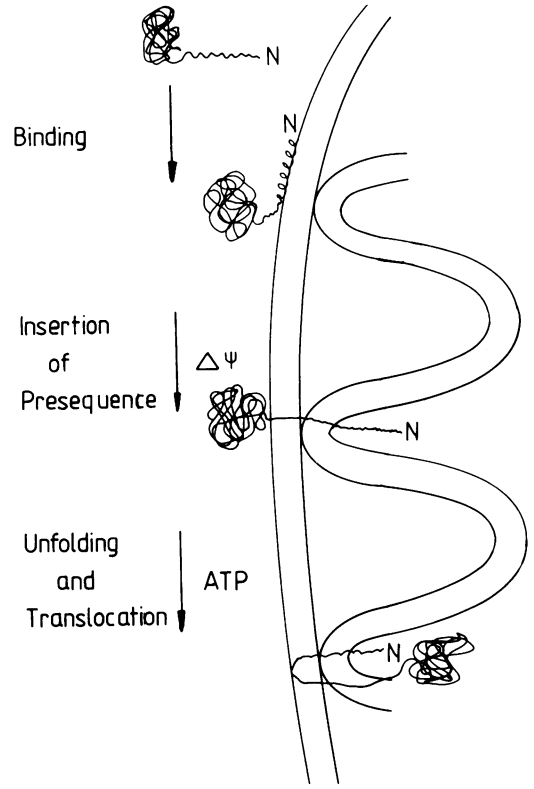

Fig. 5. Working hypothesis on the energy-requiring steps during import of the DHFR-derived fusion protein into mitochondria. Import is assumed to occur at contact sites between the two mitochondrial membranes (Kellems *et al.*, 1975; Schleyer and Neupert, 1985).

synthesis became absolutely dependent on externally-added ATP (lanes 7–10) and this effect of added ATP was strongly inhibited by carboxyatractyloside (compare lanes 8 and 10). These control experiments demonstrated that the blocks exerted by carboxyatractyloside and efrapeptin were sufficiently tight to inhibit, at least partially, an effect of added ATP on a slow reaction inside the inner membrane.

Protein import behaved differently: the combination of carboxyatractyloside and efrapeptin failed to block the stimulatory effect of added ATP (Figure 4B, compare lanes 8 and 10). This implied that ATP supported protein import by acting outside the inner membrane. This explanation, however, cannot explain why pre-incubation of mitochondria with carboxyatractyloside or with efrapeptin stimulated or inhibited, respectively, protein import in the absence of added ATP. We suspect that these inhibitors may influence the stability of mitochondria during pre-incubation in the absence of ATP. Although these effects remain puzzling, they appear to be less significant than the complete inability of carboxyatractyloside to block the effect of ATP on protein import (compare lanes 8 and 10). While we cannot exclude that ATP is also needed inside the inner membrane, its major effect is clearly outside that membrane.

Discussion

Our results show that protein import into mitochondria requires not only an energized inner membrane, but also ATP. This observation adds further credence to the suggestion (Schatz, 1986) that protein translocation across different biological membranes occurs by a basically similar mechanism: ATP is also needed for protein translocation across the bacterial plasma membrane, the endoplasmic reticulum, and the chloroplast envelopes. Protein uptake by peroxisomes (which is as yet insufficiently characterized; see Lazarow and Fujiki, 1985) may also prove to require ATP.

The mechanism by which ATP supports protein translocation remains open. One important clue is the observation that non-cleavable ATP analogs are inactive in mitochondria as well as in the other systems mentioned above. Perhaps ATP serves as an energy source for a membrane-bound 'protein translocase' which couples the hydrolysis of ATP to the transmembrane movement of polypeptide chains. Alternatively, ATP could serve as a phosphoryl donor for the phosphorylation of a receptor or a translocator, thereby activating these components for protein transport. Yet another possibility would be that ATP is needed to influence the conformation of the protein destined for transport. Evidence has been presented that only loosely folded proteins are exported from bacteria (Randall and Hardy, 1986) and that stabilization of a folded conformation of a mitochondrial precursor protein inhibits its import (Eilers and Schatz, 1986). Could protein translocation across membranes involve ATP-driven 'unfoldases', as suggested by Rothman and Kornberg (1986)? If so, then different precursor proteins may differ in their ATP requirement for transport depending on how tightly folded they were before transport. However, it is difficult to envisage a role for such an 'unfoldase' during co-translational protein translocation in which the protein to be translocated may not have the chance to fold before it is translocated.

Models invoking a role of ATP in protein unfolding and in protein chain translocation need not be mutually exclusive, however. Indeed, these phenomena may well be mechanistically coupled.

Figure 5 represents a working hypothesis on the roles of the two types of energy sources in the import of proteins into mitochondria. This model incorporates the finding that the membrane potential mediates the insertion of the (usually amino-terminal) targeting sequence into the mitochondrial inner membrane (Schleyer and Neupert, 1986). It suggests that ATP is required for unfolding/translocation of the attached 'mature' sequence.

After completion of this manuscript, a paper by Pfanner and Neupert (1986) appeared which reported that protein import into *Neurospora* mitochondria required both ATP (or GTP) and a membrane potential. Their results and conclusions are in complete agreement with ours.

Materials and methods

Isolation of mitochondria and import assays

Mitochondria were isolated from the yeast strain D 273-10B as described previously (Daum *et al.*, 1982). The artificial precursor protein was purified from an *E. coli* strain harboring the expression plasmid pKK223–pCoxIV-DHFR as before (Eilers and Schatz, 1986)

In each import assay, 100–200 μg of mitochondrial protein was incubated with 15 μl (7000 c.p.m./μl; specific radioactivity = 3×10^8 c.p.m./mg) of purified precursor protein in 0.6 M sorbitol, 20 mM Hepes–KOH, pH 7.4, 40 mM KCl, 8 mM methionine, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM succinate and 10 mM L-malate. The import assays were carried out in a final volume of 200 μl in 15 ml flat-bottomed plastic scintillation vials in a shaking waterbath to ensure sufficient aeration of the mitochondrial suspension. After 10 min at 30°C, mitochondria were re-isolated and analyzed for imported radiolabeled DHFR as described previously (Eilers and Schatz, 1986).

Inhibitors were used, where indicated, at the following final concentrations: KCN, 1 mM; efrapeptin, 50 μg/ml; oligomycin, 50 μg/ml; carboxyatractyloside, 0.2 mg/ml; valinomycin, 10 μg/ml. All concentrations were shown to be higher than those giving maximal inhibition of the respective function (M. Eilers and K. Verner, unpublished). In all experiments except those shown in Figure 1, efrapeptin was used to inhibit mitochondrial ATP synthase; efrapeptin, in contrast

to oligomycin, is freely water soluble at concentrations needed for efficient inhibition.

Potential measurements

The potential measurements were carried out with the potential-sensitive dye, (3,3′)-dipropylthiocarbocyanine iodide, (diS-C_3-(5); Sims *et al.*, 1974). Excitation was at 620 nm and emission at 670 nm. All incubations were at room temperature in 10 mM $MgCl_2$, 0.5 mM EDTA, 20 mM KPi, pH 7.4, 0.6 M sorbitol and 1 mg/ml bovine serum albumin. A 2 mM solution of the dye in dimethylsulfoxide was diluted 1000× into the incubation. Mitochondria were used from a 10 mg/ml stock solution; the final concentration in the assay was 100 μg protein/ ml. The concentration of succinate and L-malate were 5 mM each. All other additions were done at the same concentrations as in the import assays.

Mitochondrial protein synthesis

Protein synthesis by isolated mitochondria was measured essentially as described (Ohashi and Schatz, 1980) with the following modifications. The assay buffer was 60 mM sorbitol, 150 mM KCl, 3 mg/ml bovine serum albumin, 10 mM succinate, 10 mM L-malate,10 mM KPi, 10 mM α-ketoglutarate, 50 μM GDP, 10 mM $MgCl_2$ and 100 μg/ml cycloheximide. Where indicated, 1 mM ATP was added. Each assay contained 150 μg mitochondrial protein in a final volume of 200 μl. Incubations were at 30°C, the same temperature as that used for the import assays. After 5 min, labeling was started by the addition of 25 μCi of [^{35}S]methionine (1000 Ci/mmol, 10 mCi/ml) and continued for 30 min. Then, 5 μmol of non-radioactive methionine was added and the incubation continued for another 10 min. Mitochondria were then re-isolated through a 25% (w/v) sucrose cushion containing 10 mM unlabeled methionine, and solubilized in SDS-sample buffer for subsequent SDS−polyacrylamide gel electrophoresis.

Materials

Efrapeptin was a generous gift from Henry A. Lardy, University of Wisconsin. The potential-sensitive dye was obtained from Molecular Probes, Inc. The ATP-analogs, β, γ-methyleneadenosine-5′-triphosphate and α, β-methyleneadenosine-5′-triphosphate were purchased from Sigma.

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