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Translocation of proteins across the mitochondrial inner membrane, but not into the outer membrane, requires nucleoside triphosphates in the matrix

(protein import/conformation/ATP/import intermediate)

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ABSTRACT **Work in several laboratories has established that import of proteins across the mitochondrial inner membrane requires an electrochemical potential across that membrane and cleavage of nucleoside triphosphate. We now show that nucleoside triphosphate must be present inside the inner membrane. In contrast, the potential-independent insertion of porin into the outer membrane requires ATP only outside the inner membrane.**

The import of cytoplasmically made proteins into the mitochondrial interior requires a potential across the inner membrane as well as ATP (1). Attempts to identify the ATP-dependent step(s) in the import pathway have led to conflicting results. An early study with whole yeast cells indicated that import of proteins across the inner membrane required ATP in the matrix (intercristal space) (2). In contrast, subsequent studies with isolated mitochondria suggested that ATP acted outside the mitochondria (3, 4), being needed for the initial interaction of precursors with the mitochondrial surface (5). However, the data did not exclude that nucleoside triphosphate (NTP) was needed in the matrix as well, as was pointed out (3).

Recent work in our laboratory has shown that yeast mitochondria maintaining a potential across their inner membrane but depleted of ATP can productively bind purified precursor protein on their surface. Upon ATP addition, the surface-bound precursor is moved into the mitochondrial matrix, where its presequence is cleaved by the matrix-localized processing protease (6). As this movement does not require an energized inner membrane, the ATP-requiring step(s) must occur after the potential-dependent interaction of the precursor with the mitochondrial import machinery. This "ATP-depletion intermediate" offered a new approach for pinpointing the location of the ATP-requiring step(s) in the mitochondrial protein import pathway.

Here we show that the ATP-dependent chase of the surface-bound translocation intermediate is blocked by carboxyatractyloside, which inhibits exchange of adenine nucleotides across the mitochondrial inner membrane (7). This result, and other data reported in this study, reaffirm our initial suggestion (2) that protein transport across the mitochondrial inner membrane requires NTP in the matrix. In contrast, insertion of porin into the outer membrane requires only ATP outside the inner membrane. A role of NTP inside the inner membrane has fundamental implications for the still unknown mechanism by which proteins are transported across both mitochondrial membranes.

MATERIALS AND METHODS

Import of Precursor Proteins into Mitochondria. Mitochondria were prepared from the *Saccharomyces cerevisiae* strain D273-10B (ATCC 25657), as described (8). Import experiments were done at 28–30°C (unless otherwise noted) for the indicated times, either with purified pCOXIV–DHFR fusion protein (9), the precursors to cytochrome b_2, or porin synthesized by transcription/translation (10). The import buffer contained 0.6 M sorbitol, 20 mM Hepes·KOH (pH 7.4), 20 mM KP_i (pH 7.4), 40 mM KCl, 8 mM unlabeled methionine, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM succinate, 10 mM malate, and fatty acid-free bovine serum albumin at 1 mg/ml, unless noted. Energy was supplied by ATP, GTP, or ADP, or by respiratory substrates succinate and malate (3). ATP depletion of reticulocyte lysates was performed with apyrase (Sigma, grade VI; ref. 11). In some instances, we also used *Escherichia coli* glycerol kinase with glycerol (12).

Formation of the ATP-Depletion Intermediate. The ATP-depletion intermediate was generated essentially according to Eilers *et al.* (6). Isolated yeast mitochondria (100 μg of protein) were incubated with purified ^{35}S-labeled pCOXIV–DHFR fusion protein (15 ng; 3×10^8 cpm/mg of protein), oligomycin (25 μg/ml), and apyrase (10 units/ml) in 200 μl (final volume) of import buffer. The suspension was agitated vigorously for 10 min at 30°C in a flat-bottomed scintillation vial to ensure adequate aeration of the suspension. Valinomycin was added to 0.5 μg/ml, and the mitochondria were pelleted in an Eppendorf centrifuge (15,000 × *g* for 5 min at 4°C). The pellet was washed gently with 100 μl of chase buffer (import buffer plus 25 μg of oligomycin per ml and 0.5 μg of valinomycin per ml) and resuspended in 200 μl of chase buffer. The "chase" reaction was initiated with ATP or a combination of other nucleoside di- and triphosphates.

Measurements and Sources. ATP was measured by the luciferase reaction (Lumit-PM, Landgraaf, The Netherlands). Protein was assayed by the BCA procedure (Pierce). Carboxyatractyloside was purchased from Sigma.

RESULTS

Two Imported Precursor Proteins, pCOXIV–DHFR and Pre-cytochrome b_2, Require Internal ATP for Translocation. Most of our *in vitro* import studies used a radiochemically pure fusion protein (pCOXIV–DHFR) consisting of the presequence of cytochrome oxidase subunit IV (a mitochondrial inner-membrane protein) fused to mouse dihydrofolate reductase (a cytosolic protein, refs. 9 and 13). To control ATP levels inside and outside the mitochondria, we used oligomycin or efrapeptin (to block mitochondrial ATP synthase and, thus, prevent ATP from being formed internally); carboxyatractyloside (to inhibit ATP/ADP translocator and ATP movement across the mitochondrial inner membrane); and ATP scavenging systems, such as apyrase (which cleaves ATP and GTP to AMP and GMP, respectively) or *E. coli*

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Abbreviation: NTP, nucleoside triphosphate.

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glycerol kinase plus glycerol (which removes the terminal phosphate only from ATP and not other NTPs).

In the presence of carboxyatractyloside, apyrase, and the respiratory substrate succinate, ATP levels in the matrix space were 10-fold higher than after incubation with either apyrase alone or with apyrase, carboxyatractyloside, and oligomycin (Table 1; see ref. 14). This result showed that carboxyatractyloside effectively spares ATP in the matrix, even with external apyrase.

When ATP concentration outside the mitochondria was lowered to 6 nM or below, import of the pCOXIV–DHFR fusion protein was not significantly inhibited, provided the mitochondria could synthesize and accumulate ATP internally (Fig. 1 *a* and *b*). Very little, if any, external ATP is thus required for import to occur. When accumulation of intramitochondrial ATP was blocked as well, import was severely inhibited (Fig. 2, lane 5). This result indicated that ATP (or some NTP made from it) must be in the matrix for import to occur. The low import activity of mitochondria that could neither synthesize nor import ATP was weakly stimulated by external ATP (Fig. 2, lanes 7 and 8); this may suggest that NTP has some effect outside mitochondria. However, ATP added to the carboxyatractyloside-treated mitochondria could enter the matrix by a slow, carboxyatractyloside-insensitive pathway (7).

A similar result was obtained with the authentic precursor of cytochrome b_2, which had been synthesized in a reticulocyte lysate: when ATP levels outside the mitochondria were lowered from 280 μM to 32 nM, the rate of import was not decreased (Fig. 3). In a separate experiment, depletion of intramitochondrial ATP by efrapeptin with carboxyatractyloside and apyrase yielded results similar to those for the pCOXIV–DHFR fusion protein (data not shown). Thus, the major NTP requirement for translocation of an authentic and an artificial precursor protein is inside the inner membrane.

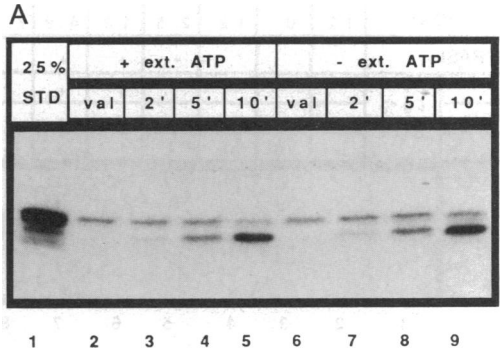
Carboxyatractyloside Blocks the ATP-Dependent Translocation of the Surface-Bound ATP-Depletion Intermediate. In the previous experiments, inhibition of import with carboxyatractyloside, apyrase, and oligomycin was not complete—probably because carboxyatractyloside effectively spares enough ATP in the matrix to allow minimum import to occur during a relatively long incubation. Therefore, we used the ATP-depletion intermediate because the translocation step for this intermediate is ≈50-fold faster than the complete import reaction (P. Scherer, personal communication). We generated the intermediate by presenting purified pCOXIV–DHFR fusion protein to respiring yeast mitochondria depleted of NTP by oligomycin and apyrase (6). The mitochondria were then reisolated, suspended with oligomycin and the uncoupler valinomycin, and "chased" by adding ATP with or without carboxyatractyloside. Without carboxyatractyloside, a large fraction of the surface-bound intermediate was imported and cleaved; this movement was almost completely blocked by carboxyatractyloside, which inhibits entry of added ATP into the matrix (Fig. 4).

ATP plus carboxyatractyloside could alter the conformation or the submitochondrial location of the translocation intermediate without cleaving the presequence. To exclude this explanation, we tested protease-sensitivity of the ATP-depletion intermediate before and after incubation with ATP plus carboxyatractyloside. Our earlier results had shown that the surface-bound intermediate is more sensitive to protein-

Table 1. Internal ATP levels

Exp.	Addition(s) to mitochondria	ATP, pmol/mg of protein
A	None	142
B	Apyrase	170
C	CAT + apyrase	1705
D	Apyrase + oligomycin + CAT	162

CAT, carboxyatractyloside. Internal mitochondrial ATP levels under different conditions. Seventy-five micrograms of yeast mitochondria per assay were incubated in 250 mM sucrose/4 mM KCl/1 mM $MgCl_2$/12.5 mM K_{Pi}/0.5 mM EDTA/2.5 mM succinate/1 mg of fatty acid-free bovine serum albumin per ml for 3 min at 30°C in a well-aerated scintillation vial with the following additions: experiment A, none; experiment B, apyrase (10 units/ml); experiment C, carboxyatractyloside (200 μg/ml) added before apyrase (10 units/ml); experiment D, oligomycin (25 μg/ml) plus apyrase (10 units/ml) added to mitochondria (5 min, 4°C) before carboxyatractyloside (200 μg/ml). After incubation, the mitochondrial suspension (≈200 μl per assay) was pipetted into a 0.5-ml polypropylene tube containing 30 μl of 10% $HClO_4$ and 100 μl of silicone oil (Wacker Chemie, Munich; AR 20/AR 200, 8:92) [modified from a procedure by Heldt *et al.* (14)]. The mitochondria were pelleted by centrifugation (1 min at 10,000 × *g*). The tube was then frozen, and the tip containing perchloric acid was removed with a razor and shaken vigorously in 500 μl of H_2O. The protein precipitate was separated by centrifugation (10,000 × *g*, 3 min), and the supernatant was neutralized by 3 M KOH and brought to pH 7.7 by adding 0.1 vol of 1 M Hepes·KOH, pH 7.7. ATP levels were then measured by using the Lumit-PM kit (see *Materials and Methods*).



B

time (min)	External ATP Levels (uM)	
	+ ATP	+ apyrase
1	247	0.006
10	198	<0.001

FIG. 1. Mitochondria efficiently import the pCOXIV–DHFR fusion protein even when external ATP levels are very low. (*A*) Aliquots (100 μg) of yeast mitochondria in import buffer were incubated with carboxyatractyloside (150 μg/ml) either with 250 μM ATP (+ ext. ATP) or apyrase at 10 units/ml (− ext. ATP); final volume of each aliquot was 200 μl. After 5 min at 4°C, purified pCOXIV–DHFR fusion protein (12 ng) was added to each aliquot, and incubation was continued for indicated times at 30°C in shallow scintillation vials. Import was stopped with valinomycin at 5 μg/ml, and the samples were placed on ice. Mitochondria were reisolated by 1.5-min centrifugation at 15,000 × *g* at 4°C, solubilized in SDS-sample buffer and analyzed by SDS/12.5% PAGE and fluorography. (*B*) External ATP levels were measured after 1 and 10 min (see *A*) by sedimenting the mitochondria by centrifugation for 1.5 min at 15,000 × *g* at 4°C, mixing 100 μl of the supernatant with 200 μl of 0.5 M $HClO_4$, and incubating the mixture for 10 min on ice. Precipitated protein was removed by centrifugation for 10 min at 4°C at 15,000 × *g* in an Eppendorf centrifuge. Protein-free supernatant was neutralized with 3 M KOH and then mixed with 0.1 vol of 1 M Hepes·KOH, pH 7.7. ATP was assayed by the luciferase reaction.

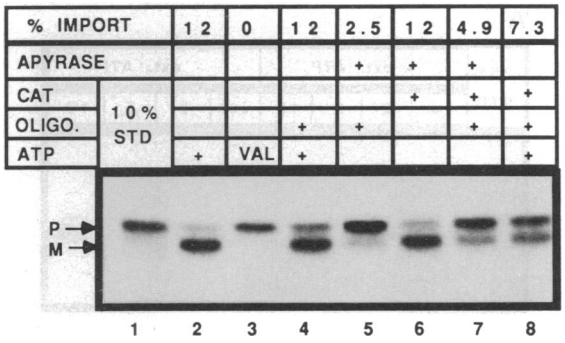


FIG. 2. Import of pCOXIV–DHFR fusion protein under different levels of internal and external ATP. Isolated yeast mitochondria (100 μg per assay) were incubated with purified pCOXIV–DHFR fusion protein (15 ng) for 7 min at 30°C in import buffer (200 μl, final volume) in shallow scintillation vials to ensure adequate aeration. Additions were made to the mitochondria just before mixing them with purified fusion protein. Final concentrations were as follows: ATP, 2.5 mM; carboxyatractyloside (CAT), 100 μg/ml; oligomycin (OLIGO), 25 μml; apyrase, 10 units/ml; valinomycin (VAL), 5 μg/ml; 10% STD, 10% of fusion protein added to each import assay. Number above each import lane indicates the percentage import [mature (M) form] of added precursor (P) measured by scanning densitometry. Because generation of the smaller, processed (M) form of the fusion protein depended on membrane potential (lane 3), it accurately measured import under these conditions.

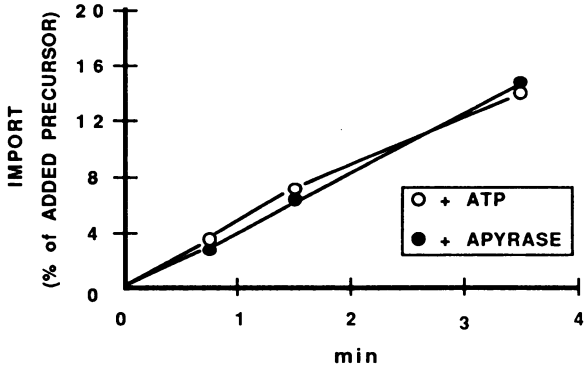


FIG. 3. Depletion of external ATP does not significantly affect import of an authentic mitochondrial precursor. Aliquots of yeast mitochondria (100 μg) were incubated with carboxyatractyloside at 100 μg/ml and either 250 μM ATP or apyrase at 10 units/ml (as for Fig. 1A) and then presented with radiolabeled pre-cytochrome b_2 synthesized in a reticulocyte lysate. The lysate added to the apyrase-treated mitochondria was pretreated with apyrase at 50 units/ml for 10 min on ice to deplete NTP. Import proceeded at 17°C for indicated times and was stopped by adding valinomycin to 5 μg/ml. Samples were treated with 25 μg of trypsin per ml for 10 min on ice and assayed by SDS/12.5% PAGE, fluorography, and densitometric scanning. Import was assessed by the sum of protease-inaccessible mature and intermediate forms of cytochrome b_2. External ATP levels after 1.5 min (as measured by luciferase assay) were 280 μM (+ ATP) and 32 nM (+ APYRASE).

ase K than the tightly folded, native precursor (6). ATP added with carboxyatractyloside changed neither the accessibility nor the sensitivity of the surface-bound intermediate toward added proteinase K (Fig. 4). This fact confirms our report (6) that partial unfolding of the pCOXIV–DHFR protein on the surface of respiring mitochondria requires little or no ATP. The major NTP-requiring step in the import of the purified pCOXIV–DHFR fusion protein is, thus, located inside the inner membrane.

GTP Must Be Converted to ATP to Support Protein Import into Mitochondria. The NTP requirement of protein import into mitochondria can be met by adding either ATP or GTP (3, 4). However, GTP could exert its effect via generation of ATP from residual ADP (GTP + ADP ⇌ ATP + GDP) by nucleoside diphosphate kinase located in the mitochondrial intermembrane space (4). Indeed, when mitochondria were treated with high levels of apyrase during formation of the ATP-depletion intermediate to degrade all external ATP and ADP, added GTP, either by itself (data not shown) or with the ATP-specific trap glycerol kinase plus glycerol (Fig. 5; lane 3) was ineffective in chasing the ATP-depletion intermediate into the mitochondria. ADP alone was effective because adenylate kinase converted some of it to ATP (lane 4), which then entered the mitochondria via the ADP/ATP translocator. The effect of ADP was blocked by the added ATP trap, which efficiently competed with adenylate kinase and lowered the ATP level by >2-fold (lane 5). However, combination of GTP and ADP was effective even with the ATP trap (lane 6) because generation of ATP by nucleoside diphosphate kinase in the intermembrane space was sufficiently rapid to compete with the ATP trap. In this case, ATP concentration was roughly 3-fold and 5-fold higher, respectively, than after addition of either GTP or ADP alone with the ATP trap (compare total ATP in lane 6 vs. lanes 3 and 5). Stimulation by GTP plus ADP was inhibited by carboxyatractyloside (lane 7), suggesting that this effect was mediated by ATP that was transported across the inner membrane to the matrix space. Fig. 4 also shows that high levels of ATP cannot drive import unless the ATP is transported into the matrix (lane 8).

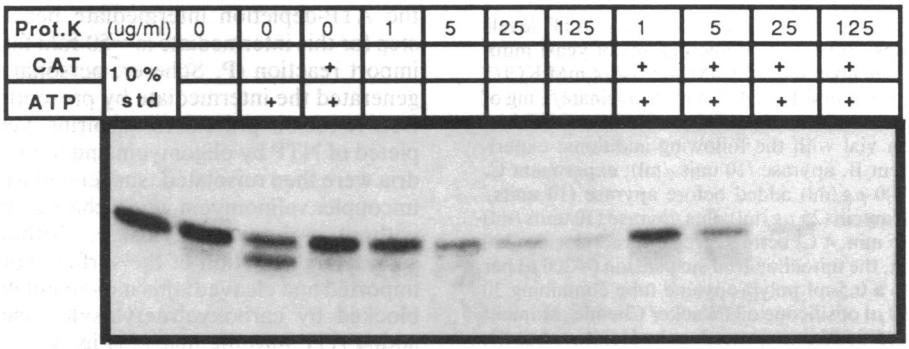


FIG. 4. Carboxyatractyloside (*CAT*) blocks the ATP-dependent import of a surface-bound pCOXIV–DHFR translocation intermediate. The ATP-depletion intermediate was generated by incubating pCOXIV–DHFR fusion protein with respiring ATP-depleted mitochondria. Mitochondria containing the bound intermediate were resuspended in ≈200 μl of the chase buffer, which included valinomycin; CAT was added to 150 μg/ml in the indicated lanes. The chase reaction was started by adding ATP to 250 μM; proteinase K (Prot. K) was then added to the indicated concentrations, and all samples were incubated for 5 min at 10°C. After adding phenylmethylsulfonyl fluoride to 1 mM, we isolated the mitochondria by centrifugation at 15,000 × *g* for 5 min and analyzed the results by SDS/12.5% PAGE and fluorography. Under these conditions, cleavage of the precursor upon ATP addition is a valid measure of import (6).

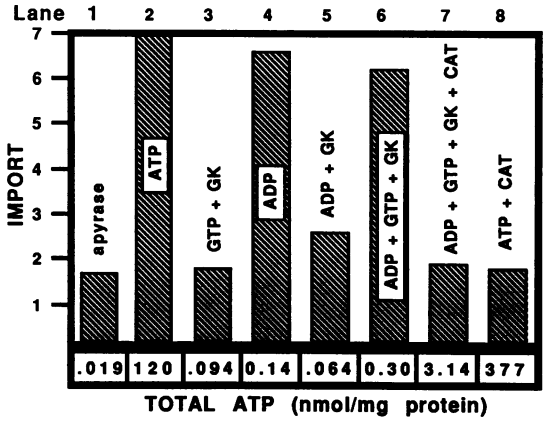


FIG. 5. Externally added GTP supports translocation of the ATP-depletion intermediate only in the presence of ADP. The ATP-depletion intermediate was generated as for Fig. 4, and mitochondria were resuspended in chase buffer. Where indicated, glycerol kinase plus glycerol (GK) was added to 6 units/ml and 50 mM, respectively, carboxyatractyloside (CAT) was added to 200 μg/ml, and apyrase was added to 10 units/ml. The chase reaction was then started by adding ATP, GTP, or ADP to 500 μM, 500 μM, or 33 μM. After 4 min at 20°C, apyrase was added to 5 units/ml; mitochondria were pelleted by centrifugation and analyzed by SDS/12.5% PAGE, fluorography, and densitometric scanning of the imported mature form. Total ATP in the suspension was measured as described above. Two minutes after initiation of the chase reaction, the mitochondrial suspension in a final volume of 200 μl was added to 400 μl of 0.5 M $HClO_4$. After centrifugation, the supernatant was neutralized with KOH and assayed for ATP as for Fig. 1*B*. Import values on the ordinate are in arbitrary scanner units.

Porin Requires External ATP for Insertion into the Mitochondrial Outer Membrane. When porin (the major pore-forming protein of the outer membrane) is synthesized in a reticulocyte lysate, it requires ATP for its proper insertion into the outer membrane of intact mitochondria (15). This ATP requirement could be fully met by externally added ATP under conditions in which intramitochondrial generation of ATP, as well as ATP import into the matrix, was blocked by KCN and carboxyatractyloside (Fig. 6). As noted earlier (16), this insertion was also little affected by collapsing the potential across the inner membrane by KCN or valinomycin. For comparison, Fig. 6 also depicts the import of pCOXIV–DHFR, which, as noted above, was not inhibited when external ATP was lowered to nanomolar concentrations. When mitochondria and lysate had been pretreated with glycerol kinase plus glycerol, GTP did not support the insertion of porin (data not shown). Thus, insertion of this protein into the outer membrane specifically requires ATP outside the inner membrane.

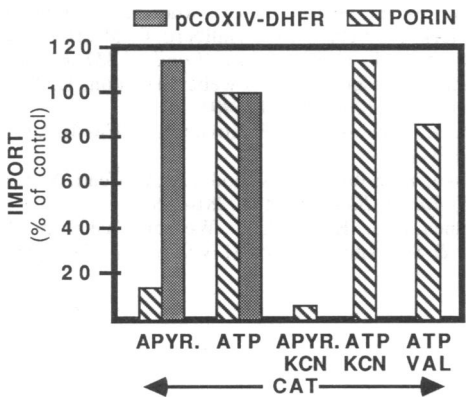


FIG. 6. Insertion of porin into the outer membrane requires only external ATP. Yeast mitochondria (100 μg for pCOXIV–DHFR import and 20 μg for porin import) were incubated with carboxyatractyloside (CAT) at 200 μg/ml and, where indicated, with 1 mM KCN or valinomycin at 0.5 μg/ml. Apyrase (APYR.) (10 units/ml) or ATP (125 μM) was then added to the indicated samples. Import was measured either with yeast porin synthesized in a reticulocyte lysate or with purified pCOXIV–DHFR fusion protein. Lysate added to apyrase-treated mitochondria was pretreated for 10 min at 20°C with apyrase at 50 units/ml. Import was measured at 30°C for 7 min (pCOXIV–DHFR) or 10 min (porin). Under those conditions, import of both precursors was linear with time. Import was stopped by adding (to all samples) valinomycin (0.5 μg/ml) and apyrase (10 units/ml) followed by 20-min incubation on ice with proteinase K (200 μg/ml) to remove nonimported precursor protein. After adding phenylmethylsulfonyl fluoride to 1 mM, mitochondria were reisolated and analyzed by SDS/12.5% PAGE, fluorography, and densitometric scanning of the fluorograms. Import was normalized to import with CAT and added ATP, which was taken as 100%; this value represented 15% import of added pCOXIV–DHFR and 30% import of added porin.

DISCUSSION

In all membrane systems studied so far, ribosome-independent transport of proteins requires NTP cleavage (17). To understand how NTPs act, it is essential to know on which side of the membrane barrier they are cleaved. In chloroplasts, NTPs must be present in the stroma to allow transport of cytoplasmically made proteins across the two envelope membranes (18, 19), although they may also be required outside the chloroplasts (20) to facilitate binding of precursors to the surface of these organelles (21). In *E. coli*, transport of proteins into or across the plasma membrane is mediated by cleavage of ATP by the SecA protein on the cytosolic face of that membrane (22). For mitochondria, evidence on the site of action of NTPs has been conflicting. We now show that protein transport across the mitochondrial inner membrane requires NTP in the matrix (i.e., on the trans-side of the membrane), whereas insertion of porin into the outer membranes requires ATP on the outside of the inner membrane.

Until now, an action of NTP outside the mitochondria was mainly inferred from the fact that added ATP stimulated protein import into ATP-depleted mitochondria, even in the presence of carboxyatractyloside, which blocks the exchange of adenine nucleotides across the inner membrane. This carboxyatractyloside-insensitive stimulation was confirmed in the present work (Fig. 2, lanes 7 and 8). However, protein import into isolated mitochondria is relatively slow; this result left open the possibility that the carboxyatractyloside-insensitive stimulation might be caused by ATP that entered mitochondria by a minor carboxyatractyloside-insensitive pathway (7). To minimize complications arising from such a bypass, we have now specifically studied the ATP-dependent step of protein import. The overall reaction consists of an early step that is slow and NTP-independent and a late step that is fast and NTP-dependent. Our present data show that the effect of added ATP on this second step is completely blocked by carboxyatractyloside (Fig. 4). This block does not reflect some side effect of carboxyatractyloside because carboxyatractyloside actually stimulates import when internal ATP production is limited by oligomycin (Fig. 2, lane 5 vs. 7) or when mitochondria are incubated in the presence of apyrase. This stimulation agrees with the fact that carboxyatractyloside raises intramitochondrial ATP levels by preventing loss of ATP to the outside (Table 1).

That import into the matrix is supported by NTP that has diffused through the outer membrane pores into the intermembrane space can be excluded. If this occurred, carboxyatractyloside should not inhibit the ATP-dependent chase of the translocation intermediate; in fact, carboxyatractyloside inhibits it completely.

Although exogenously added GTP must be converted to ATP outside the mitochondria to support the NTP-dependent chase (Fig. 5), the type of NTP active within the matrix is

unknown. However, insertion of porin into the outer membrane is supported by ATP, but not by GTP.

How can NTP in the matrix support protein import across both mitochondrial membranes? It could displace newly imported precursor proteins from a chaperone protein or "chaperonin" (23) in the matrix, thereby freeing this chaperone molecule for another round of catalysis. However, the ATP-depletion intermediate is apparently not in direct contact with the matrix (6), and inactivation of the chaperone heat shock protein 60 (HSP60) does not block import (24). We, therefore, favor the possibility that the transmembrane machinery that imports protein into the matrix space includes a component with an NTP-cleaving domain on the matrix-side of the inner membrane. This possibility would be analogous to bacterial systems catalyzing the ATP-dependent uptake of molecules from the outside into the cytoplasm. It also agrees with our finding that ATP-dependent uptake of precursor proteins is observed with inner membrane vesicles essentially free of outer membranes (25). These vesicles may prove helpful in identifying the component(s) interacting with NTP during protein transport into mitochondria.

The results presented here may provide an explanation for the observation by Kováč and coworkers that yeast cells depleted of intramitochondrial ATP may lose mitochondrial genes (26) and stop growing (27) even when they generate ATP in the cytoplasm.

Recent evidence indicates that 70-kDa stress proteins are involved in the translocation of some precursors; these data suggest that mitochondrial protein import *in vivo* requires cytosolic ATP (28). Our present data do not contradict this possibility; they do show, however, that the NTP requirement of the actual translocation process is predominantly met by NTP in the matrix.

We gratefully acknowledge valuable help by Dr. Roy McCauley in the experiments on porin import, by Dr. K. Mihara (Kyushu University, Japan) in providing an expression plasmid for yeast porin, by T. Jascur for providing purified pCOXIV–DHFR fusion protein, by Dr. W. Ziemke in measuring intramitochondrial ATP concentrations, and by Barbara Tschopp in preparing the manuscript. This study was supported by a predoctoral fellowship (to S.H.) from the Sarnoff Endowment for Cardiovascular Science and by Grants 3.335-0.86 from the Swiss National Science Foundation and CBY-1 1 ROI GM 37803 from the U.S. Public Health Service.

1. Eilers, M. & Schatz, G. (1988) *Cell* **52,** 481–483.
2. Nelson, N. & Schatz, G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4365–4369.
3. Eilers, M., Oppliger, W. & Schatz, G. (1987) *EMBO J.* **6,** 1073–1077.
4. Chen, W. & Douglas, M. (1987) *Cell* **49,** 651–658.
5. Pfanner, N., Tropschug, M. & Neupert, W. (1987) *Cell* **49,** 815–823.
6. Eilers, M., Hwang, S. & Schatz, G. (1988) *EMBO J.* **7,** 1139–1145.
7. Klingenberg, M. (1989) *Arch. Biochem. Biophys.* **270,** 1–22.
8. Daum, G., Boehni, P. & Schatz, G. (1982) *J. Biol. Chem.* **257,** 13028–13033.
9. Eilers, M. & Schatz, G. (1986) *Nature (London)* **322,** 228–232.
10. Hurt, E. C., Pesold-Hurt, B. & Schatz, G. (1984) *EMBO J.* **3,** 3149–3156.
11. Pfanner, N. & Neupert, W. (1986) *FEBS Lett.* **209,** 152–156.
12. Hayashi, S. & Lin, E. C. C. (1967) *J. Biol. Chem.* **242,** 1030–1035.
13. Hurt, E. C., Pesold-Hurt, B. & Schatz, G. (1984) *FEBS Lett.* **178,** 306–310.
14. Heldt, H. W., Klingenberg, M. & Milovancev, M. (1972) *Eur. J. Biochem.* **30,** 434–440.
15. Pfanner, N., Pfaller, R., Kleene, R., Ito, M., Tropschug, M. & Neupert, W. (1988) *J. Biol. Chem.* **263,** 4049–4051.
16. Hartl, F.-U., Pfanner, N., Nicholson, D. W. & Neupert, W. (1989) *Biochim. Biophys. Acta* **988,** 1–45.
17. Verner, K. & Schatz, G. (1988) *Science* **241,** 1307–1313.
18. Pain, D. & Blobel, G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3288–3292.
19. Theg, S. M., Bauerle, C., Olsen, L. J., Selman, B. R. & Keegstra, K. (1989) *J. Biol. Chem.* **264,** 6730–6736.
20. Fluegge, U. I. & Hinz, G. (1986) *Eur. J. Biochem.* **160,** 563–570.
21. Olsen, L. J., Theg, S. M., Selman, B. R. & Keegstra, K. (1989) *J. Biol. Chem.* **264,** 6724–6729.
22. Lill, R., Cunningham, K., Brundage, L., Ito, K., Oliver, D. & Wickner, W. (1989) *EMBO J.* **8,** 961–966.
23. Ellis, J. (1987) *Nature (London)* **328,** 378–379.
24. Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. & Horwich, A. L. (1989) *Nature (London)* **337,** 620–625.
25. Hwang, S., Jascur, T., Vestweber, D., Pon, L. & Schatz, G. (1989) *J. Cell Biol.* **109,** 487–493.
26. Subik, J., Takacsova, G. & Kováč, L. (1978) *Mol. Gen. Genet.* **166,** 103–116.
27. Gbelska, Y., Subik, A., Svoboda, A., Goffeau, A. & Kováč, L. (1983) *Eur. J. Biochem.* **130,** 281–286.
28. Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. & Schekman, R. (1988) *Nature (London)* **332,** 800–805.