GTP Hydrolysis Is Essential for Protein Import into the Mitochondrial Matrix*

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Protein import into the innermost compartment of mitochondria (the matrix) requires a membrane potential (ΔΨ) across the inner membrane, as well as ATP-dependent interactions with chaperones in the matrix and cytosol. The role of nucleoside triphosphates other than ATP during import into the matrix, however, remains to be determined. Import of urea-denatured precursors does not require cytosolic chaperones. We have therefore used a purified and urea-denatured preprotein in our import assays to bypass the requirement of external ATP. Using this modified system, we demonstrate that GTP stimulates protein import into the matrix; the stimulatory effect is directly mediated by GTP hydrolysis and does not result from conversion of GTP to ATP. Both external GTP and matrix ATP are necessary; neither one can substitute for the other if efficient import is to be achieved. These results suggest a "push-pull" mechanism of import, which may be common to other post-translational translocation pathways.

Most (>95%) mitochondrial proteins are synthesized on cytoplasmic ribosomes. These proteins must be imported from the cytosol into the correct mitochondrial subcompartment (outer membrane, intermembrane space, inner membrane, or matrix). Import of preproteins into the innermost compartment (the matrix) is achieved by the coordinated action of two independent translocases; one in the outer and another in the inner membrane (1–4). A fundamental question is what drives the translocation of proteins into the matrix across two (outer and inner) membrane barriers. This process requires a membrane potential (ΔΨ) across the inner membrane and matrix ATP (5–18). The membrane potential (negative inside) is required for the initial insertion and partial translocation of the N terminus of the precursor containing a positively charged signal sequence across the inner membrane. Subsequent translocation is completed by ATP-dependent interactions in the matrix.

The mechanisms of protein translocation across various membranes apparently share some key features (19, 20). One of the most striking examples is the participation of GTP in vesicular trafficking and protein trafficking to nucleus, endoplasmic reticulum, and chloroplasts (for review see Refs. 21–24). However, so far there is no evidence that nucleoside triphosphates (NTPs) other than ATP have any direct role in targeting a precursor to the mitochondrial surface, providing energy for transmembrane movement, or for transient unfolding of preproteins en route to the matrix (5–10, 19).

Mitochondrial import of preproteins synthesized in cell-free translation systems often requires cytosolic ATP-dependent interactions with chaperones (25–29). Consequently, the requirement of external ATP for import of native preproteins may not be bypassed. Due to the required presence of ATP, it has been difficult to determine the individual role of NTPs during the import process. Import of denatured precursors, however, does not require cytosolic chaperones (30–33). Therefore to investigate the GTP requirements for protein import into the mitochondrial matrix, we used a purified, urea-denatured precursor (delta-1-pyrroline-5-carboxylate dehydrogenase, pPut) in our assay. We demonstrate that the translocation of pPut into the matrix is stimulated by GTP. The stimulatory effect by GTP is direct; it requires energy released by GTP hydrolysis and does not result from conversion of GTP to ATP. Our data strongly suggest that the mitochondrial translocation apparatus is powered by an external “push” mediated by GTP hydrolysis and an internal ATP-dependent “pull”. Both are necessary and neither one can substitute for the other.

EXPERIMENTAL PROCEDURES

For the preparation of radiolabeled pPut, Escherichia coli BL21(DE3) cells carrying the plasmid pNYHM170 were induced by isopropyl-β-D-thiogalactopyranoside in the presence of [35S]Met and subsequently purified as described (34). Mitochondria were isolated from Saccharomyces cerevisiae strain D273-10B (ATCC 26457) (28). Import assay buffer consisted of HSB (20 mM Hepes-KOH, pH 7.5, 0.6 M sorbitol, 0.1 mg/ml bovine serum albumin) containing 40 mM KAc, 10 mM Mg(OAc)2, 5 mM unlabeled Met and 1 mM dithiothreitol. Import was initiated by adding urea-denatured [35S]M-labeled pPut (75 ng) to mitochondria (100 µg) in the assay buffer. The final urea concentration was 0.16 M. A final urea concentration as high as 0.8 M does not inhibit import of native precursors (30). Following import, reaction mixtures were treated with trypsin (0.1 mg/ml) for 30 min on ice. Samples were diluted with HSB containing 5 mg/ml soybean trypsin inhibitor, 100 units/ml Trasylol, and 1 mM phenylmethylsulfonyl fluoride. Mitochondria were sedimented and washed with 10% trichloroacetic acid. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Bands (pPut and mPut) were quantitated using the software NIH Image.

Import of pPut (64 kDa) into isolated mitochondria was assayed by the following criteria: (i) cleavage of the signal sequence (~3 kDa) by matrix-localized signal peptidase, (ii) protection of the signal-less mature polypeptide (mPut, ~61 kDa) from digestion by an exogenous protease, and (iii) sedimentation of imported and protected molecules with mitochondria upon centrifugation. Import efficiency was calculated as the percent conversion of pPut to mPut as described (28). The ladder

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1 The abbreviations used are: NTP, nucleoside triphosphate; pPut, delta-1-pyrroline-5-carboxylate dehydrogenase precursor; mPut, mature form of pPut; αKG, α-ketoglutarate; PAGE, polyacrylamide gel electrophoresis; NDP, nucleoside diphosphate; GTPyS, guanosine 5′-3′-O-(thio)triphosphate.
of bands below mPut (Figs. 1–4) are likely to result from the partial cleavage of molecules in transit to the matrix. These ladders are not the result of incomplete digestion of nonimported molecules by trypsin, since (i) when import was inhibited in the presence of valinomycin (which dissipates the ΔΨ across the inner membrane), pPut was completely digested by trypsin, and (ii) both precursor and mature forms of Put were completely digested by trypsin in the presence of Triton X-100 (not shown).

**RESULTS**

**Kinetics of pPut Import**—We examined the nucleotide requirements for import of urea-denatured pPut into the mitochondrial matrix at various temperatures. This is particularly important for two reasons. First, a wide range of import incubation temperatures and times have been reported in the literature. A thorough study on import kinetics, however, is lacking. Second, previous studies have concluded that GTP must be converted to ATP, presumably by nucleoside diphosphate kinase (NDP kinase) located in the intermembrane space (6), to support protein import (9). Such an enzymatic exchange process is expected to be temperature-dependent. The nucleotide specificity of mitochondrial protein import needs, therefore, to be tested at various temperatures.

The data presented in Fig. 1 clearly demonstrate that regardless of the temperature GTP-mediated import was always much greater (2.4–50-fold) than the corresponding ATP-mediated import. Should GTP be converted to ATP to drive import, it is very unlikely that such a conversion would ever result in import efficiency greater than that obtained by an equivalent amount of externally added ATP. Furthermore, the absolute import efficiency in GTP-containing samples were significant, ranging from 14.3% (0 °C, 30 min, Fig. 1A, lane 11) to 36.1% (30 °C, 10 min, Fig. 1D, lane 11). On the other hand, ATP could drive import to levels above 5% only at 30 °C but not at any lower temperature that we tested. Interestingly, after 5 min at 30 °C, GTP-mediated import reached a plateau, whereas ATP-
mediated import was in the early stage of the ascending linear range (Fig. 1D). With longer incubation (∼20–30 min) at 30 °C, the difference between GTP- and ATP-mediated import was much reduced (not shown). This could be due to the slow conversion of ATP, presumably by NDP kinase located in the intermembrane space (6), to GTP (i.e. ATP + GDP ⇌ ADP + GTP). This might explain why some of the earlier studies, where import incubations were longer and carried out at higher temperatures, failed to detect the GTP-stimulatory effect over that mediated by ATP (5–8). We conclude that (a) GTP is required for import of pPut into the matrix, and (b) GTP need not be converted to ATP to exert its stimulatory effect on import.

Unlike preproteins synthesized in reticulocyte lysate, urea-denatured pPut was imported even on ice (Fig. 1A). Furthermore, import of denatured pPut did not require addition of cytosolic chaperones. Similar results were previously reported using other precursors (30–33). The urea-denatured precursor circumvents at least one highly temperature-sensitive and rate-limiting step, which presumably represents the unfolding of the native precursor prior to import into isolated mitochondria (30). Subsequent experiments to monitor the nucleotide requirements for import of denatured pPut into the matrix were therefore performed at low as well as physiologically relevant temperatures.

**GTP Is the Preferred Source of External Energy**—We titrated (Fig. 2) the requirements of GTP (50 μM–2 mM, lanes 2–7) and ATP (100 μM–2 mM, lanes 8–12) for import of pPut. Considerable import was observed even on ice with physiological concentrations of GTP (∼100 μM, (35)), and little further dose-dependent stimulation was observed beyond 200 μM (Fig. 2A). Thus, the stimulation of mitochondrial import by GTP is physiologically significant. In contrast, ATP was much less effective; mPut that appeared in the presence of 2 mM ATP (Fig. 2A, lane 12) was only 65% of that obtained at one-fortieth the concentration of GTP (50 μM, Fig. 2A, lane 2). When reaction mixtures were shifted from ice to 30 °C (Fig. 2B), GTP-mediated import was further stimulated by about 50% to 2-fold. Under identical conditions, ATP-mediated import efficiency, however, was significantly increased (5–8-fold). Nevertheless, the GTP effect was higher than the corresponding ATP effect at all concentrations tested. Since the mitochondrial inner membrane is impermeable to GTP (36), these results suggest that GTP is the primary source of external energy, both at low (0 °C, Fig. 2A) and physiologically relevant temperatures (30 °C, Fig. 2B).

**Efficient Import Requires External GTP and Matrix ATP**—The complicated issue of the interconversion of NTPs by mitochondrial NDP kinase perhaps can best be resolved by using a specific inhibitor of the enzyme. Unfortunately, no such inhibitor is known. Alternatively, highly specific NTPases could be used to selectively deplete a particular NTP. For example, of all NTPs tested, only ATP can act as a phosphate donor substrate for E.coli glycerol kinase, which converts glycerol to glycerol-3-phosphate; the reaction is essentially irreversible (37). This enzyme has been used as a means of selectively degrading mitochondrial ATP while sparing other NTPs (8). This ATP-scavenging system is likely to degrade both surface-bound ATP as well as ATP in the intermembrane space. The mitochondrial outer membrane is presumably not a barrier for rapid equilibration of NTPs between the cytosol and the intermembrane space. We therefore utilized glycerol kinase to determine the NTP specificity for import in our assays.

Mitochondria were pretreated with glycerol kinase plus glycerol. The ATP trap was present during import as well. Whereas ATP-mediated import was completely abolished (Fig. 3, lanes 2 and 4), GTP-mediated import was essentially unaffected (Fig. 3, lanes 3 and 5), thereby confirming our earlier conclusion (Figs. 1 and 2) that GTP need not be converted to ATP to exert its effect. To delineate the individual role of matrix ATP and external GTP, we performed the following experiments. Mitochondria were preincubated with efrapeptin (a potent inhibitor of the F1 moiety of mitochondrial ATPase) to block respiration-driven ATP synthesis and also with glycerol kinase (8). Both efrapeptin and the ATP trap were present during the entire import reaction (Fig. 3, lanes 6–12). GTP-mediated import into these ATP-deprived mitochondria was reduced by about 60% (compare lanes 3 and 7), suggesting that both matrix ATP and external GTP are required for efficient import. Indeed, when ATP-deprived mitochondria were supplemented with α-ketoglutarate (αKG) to regenerate matrix ATP (10) through substrate-level phosphorylation via the tricarboxylic acid cycle, the inhibition was almost completely relieved (compare lanes 3, 7, and 10). In the absence of GTP, matrix ATP was completely ineffective (lane 12). How can we rule out the possibility that the generation of ATP from GTP by NDP kinase in the intermembrane space was extremely rapid, and that ATP so generated immediately entered into the matrix via the ADP/ATP carrier, thereby escaping the trap? This seems unlikely for two reasons: (a) carboxyatractysolide (which blocks the ADP/ATP carrier) did not interfere with the reversal of inhibition (compare lanes 10 and 11); and (b) no import was detected either with ATP alone, αKG alone, or αKG plus ATP (lanes 6, 8 and 9, respectively). Thus, efficient import requires both matrix ATP and external GTP; neither can replace the other.

**GTP Hydrolysis Is Essential**—To determine whether mere
Both external GTP and matrix ATP are necessary for efficient import. Mitochondria were preincubated at 10 °C for 10 min in the absence, or presence of either glycerol kinase (GK) or glycerol kinase plus efrapeptin as described (8). The final concentrations of efrapeptin and GK in the import assay were 5 μg/ml and 2.8 units/ml (1.4 units/mg mitochondrial protein), respectively. All samples contained 10 mM glycerol. Where indicated, 100 μg/ml carboxyatractyloside (CAT) was included and incubated for 5 min on ice before reaction mixtures were supplemented with either ATP (1 mM) or GTP (1 mM) or αKG (5 mM), or a combination. Import of urea-denatured pPut was carried out at 30 °C for 4 min. Samples were subjected to trypsin treatment and analyzed. The quantitative data on import efficiency are presented below the autoradiograph.

GTP binding or GTP hydrolysis is necessary for import, translocation of pPut was measured (Fig. 4) in the absence or presence of a nonhydrolyzable analog, GTPγS, either on ice (lanes 2–5) or with an additional incubation at 30 °C (lanes 6–9). Under both conditions, GTP-mediated import was strongly inhibited by GTPγS in a dose-dependent manner. GTPγS alone was completely ineffective for promoting import (not shown). These data indicate that GTP hydrolysis is essential for import into the matrix.

DISCUSSION

Although GTP plays an important role in protein trafficking across various membranes, thus far there is no evidence that GTP plays a direct role in mitochondrial protein import (for review see Ref. 19). Our data demonstrate that GTP hydrolysis does indeed play a direct, essential role in translocation of proteins into the mitochondrial matrix, and that mitochondria can now be added to the list of trafficking systems where GTP hydrolysis plays a critical role. Thus, the data presented here confirm earlier predictions that protein translocation across membranes share certain key features (19, 20).

GTP was previously found to substitute for ATP in promoting import of preproteins into the matrix (5–8). It was subsequently concluded that GTP must first be converted to ATP, presumably by the NDP kinase located in the intermembrane space (6), to support protein import (9). The ATP thus generated could enter the matrix via the ADP/ATP carrier and drive translocation. These studies were performed using ATP-depleted mitochondria (9). We found that the GTP-mediated import into apyrase-pretreated mitochondria was much lower compared with import into untreated mitochondria (not shown). It is possible that GTP might be necessary, but not sufficient, for efficient protein import. Specifically, NTPs might exert their effect only in the presence of adequate amounts of matrix ATP. Indeed, our data demonstrate that GTP need not be converted to ATP to exert its stimulatory effect on import.

Both matrix ATP and external GTP are necessary, and neither one can substitute for the other if efficient import is to be achieved. These conclusions are derived from the following results. (i) Physiological concentrations of GTP can drive significant import (Fig. 2). (ii) GTP hydrolysis is essential for import to proceed (Fig. 4). (iii) GTP-mediated import is much greater than ATP-mediated import regardless of NTP concentration, incubation temperature, or the time that we tested (Figs. 1 and 2). Depending on the conditions, GTP-mediated import is 2.4–50 times more efficient. Should GTP be converted to ATP to exert its effect on import, GTP-mediated import would be very unlikely to exceed that obtained with an identical concentration of externally added ATP. (iv) The absolute GTP-mediated import efficiency is comparable to that achieved by both ATP and GTP (not shown). (v) A specific ATP-scavenging system (like glycerol kinase and glycerol) does not inhibit GTP-mediated import (Fig. 3). (vi) GTP-mediated import into ATP-deprived mitochondria is significantly reduced but not completely abolished. Supplementing these mitochondria with αKG to regenerate matrix ATP quantitatively relieves the inhibition even in the presence of an ATP trap. Matrix ATP alone is ineffective in driving import (Fig. 3).

What, then, are the energetics of mitochondrial import? Two models have been proposed to explain how ATP can assist in pulling a polypeptide into the mitochondrial matrix (for review see Ref. 19). According to the Brownian ratchet model (38, 39), movement of the translocating chain is accomplished by Brownian motion acting in concert with the mt-Hsp70-Tim44 complex. This complex binds the incoming polypeptide chain as it emerges on the matrix side of the inner membrane channel (40–43). Since the translocating chain can oscillate randomly within the channel (44), binding of this complex on the matrix side prevents backward movement of the chain. In the translocation motor model (45), the binding of the polypeptide chain to the mt-Hsp70-Tim44 complex stimulates ATP hydrolysis by mt-Hsp70. This leads to a conformational change in mt-Hsp70,
which then actively pulls the bound precursor into the matrix. Neither of these models, however, explains the energy requirements of translocation before a sufficient length of the N-terminus has reached the matrix.

We propose that GTP hydrolysis shifts the equilibrium of polypeptide chains on the cis and trans sides of the inner membrane to one favoring a trans (matrix) location. Subsequently, mt-Hsp70-Tim44 cycles trap the incoming polypeptide. In addition to a ΔΨ across the inner membrane, a push-pull mechanism might be critical to mitochondrial protein import. GTP hydrolysis at the cis side, and ATP-dependent processes at the trans side of the inner membrane presumably constitute the respective push and pull. The energy of GTP hydrolysis could improve the fidelity of the translocation process by actively pushing the polypeptide-in-transit through the import channels or else it could help maintain the polypeptide in a translocation-competent conformation during import. Recently, two GTP-binding proteins and a Hsp70 homolog with a large domain exposed to the intermembrane space have been identified as components of the outer membrane import machinery of chloroplasts (46, 47). These GTP-binding proteins have been suggested to be involved in regulating precursor binding at the chloroplast surface (46). It is also possible that one or both of these GTP-binding protein(s), in concert with Hsp70, provide the respective push and pull for post-translational transmembrane movement of proteins across the outer membrane of chloroplasts.

Although several constituents of the mitochondrial translocation machinery have been identified (for review see Ref.48), none is reported to have a GTP-binding motif. The precise role of GTP can be determined only after identification and characterization of novel GTP-binding proteins and/or GTPases involved in mitochondrial translocation. The GTP-dependent mitochondrial translocation system described here will facilitate the identification of unknown components of the translocation apparatus and assist in revealing the mechanisms and energetics involved in the insertion and translocation of preproteins into and across mitochondrial membranes.

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