A GTP-dependent “Push” Is Generally Required for Efficient Protein Translocation across the Mitochondrial Inner Membrane into the Matrix*

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Mitochondrial biogenesis requires translocation of numerous preproteins across both outer and inner membranes into the matrix of the organelle. This translocation process requires a membrane potential (ΔΨ) and ATP. We have recently demonstrated that the efficient import of a urea-denatured preprotein into the matrix requires GTP hydrolysis (Sepuri, N. B. V., Schülke, N., and Pain, D. (1998) J. Biol. Chem. 273, 1420–1424). We now demonstrate that GTP is generally required for efficient import of various preproteins, both native and urea-denatured. The GTP participation is localized to a particular stage in the protein import process. In the presence of ΔΨ but no added nucleoside triphosphates, the transmembrane movement of preproteins proceeds only to a point early in their translocation across the inner membrane. The completion of translocation into the matrix is independent of ΔΨ but is dependent on a GTP-mediated “push.” This push is likely mediated by a membrane-bound GTPase on the cis side of the inner membrane. This conclusion is based on two observations: (i) GTP does not readily cross the inner membrane barrier and hence, primarily acts outside the inner membrane to stimulate import, and (ii) the GTP-dependent stage of import does not require soluble constituents of the intermembrane space and can be observed in isolated mitoplasts. Efficient import into the matrix, however, is achieved only through the coordinated action of a cis GTP-dependent push and a trans ATP-dependent “pull.”

Most mitochondrial proteins are synthesized as precursors in the cytosol and subsequently translocated into the organelle. Proteins targeted to various mitochondrial subcompartments (outer membrane, intermembrane space, inner membrane or matrix) follow a variety of import pathways that differ in their energy requirements. For example, a membrane potential is required for the translocation of a preprotein across the inner membrane, but not into or across the outer membrane (1–3). Translocation of proteins across both membranes into the matrix of the organelle is particularly complex. It requires the coordinated action of two separate translocases, one (Tom) located in the mitochondrial outer membrane and the other (Tim) located in the inner membrane (4–6). Also required are ATP-dependent interactions with molecular chaperones both outside and inside the organelle (for review, see Refs. 7 and 8).

Among the cytosolic chaperones are Hsp70 and mitochondrial import stimulating factor (MSF). Hsp70 generally interacts with preproteins to keep them in a translocation-competent conformation. MSF, on the other hand, specifically binds mitochondrial signal sequences, preventing and/or reversing preprotein aggregation, then targeting the precursors to the outer membrane. The MSF-dependent import pathway requires cytosolic ATP, whereas the Hsp70-dependent pathway does not (7). The import of urea-denatured precursors does not require added cytosolic chaperones and may or may not require ATP (9–12).

Matrix-localized Hsp70 (mt-Hsp70) interacts with the incoming polypeptide chain as it emerges on the matrix (trans) side of the inner membrane channel. This interaction prevents backward movement of the translocating protein. The two proposed models, “Brownian ratchet” (13, 14) and “molecular motor” (15, 16), posit that mt-Hsp70 and ATP-hydrolysis in the matrix participate in the vectorial movement of preproteins into this compartment. Mechanistic details of how mt-Hsp70 participates, however, remain unclear (for review, see Ref. 8).

Do nucleoside triphosphates (NTPs) other than ATP participate in mitochondrial protein import? Specifically, do they contribute to the driving force required for unidirectional translocation across the mitochondrial membranes? More than a decade ago, GTP was found to be capable of replacing ATP in promoting mitochondrial protein import (2, 17–19). Subsequent studies with ATP-depleted mitochondria, however, concluded that GTP must first be converted to ATP to support import of a hybrid precursor, pCOXIV-DHFR, into the matrix (20). It was argued that other NTPs exert an effect only via their conversion to ATP (NTP + ADP = NDP + ATP), presumably by nucleoside diphosphate kinase (NDP kinase) located in the mitochondrial intermembrane space (IMS). The ATP thus generated could then enter the matrix via the ADP/ATP carrier and drive translocation. As these studies were performed using ATP-depleted mitochondria, they could not rule out the possibility that GTP might exert an effect in the presence of sufficient amounts of matrix ATP, which is always required for import into the matrix (21).

We have recently demonstrated that the efficient import of urea-denatured pPut, the precursor of a soluble matrix protein...
of yeast mitochondria, is dependent upon GTP hydrolysis (22). In this report, we demonstrate that GTP significantly stimulates import of several authentic and chimeric precursors into the matrix. The GTP-mediated stimulation is independent of the structural integrity of the precursor protein to be imported; import of native as well as urea-denatured precursors is strongly stimulated by a direct participation of GTP. In intact mitochondria, proteins must cross both outer and inner membrane barriers to reach the matrix. However, when the mitochondrial outer membrane is disrupted by hypotonic shock to generate mitoplasts, translocation of proteins into the matrix can occur directly across the inner membrane (23, 24). We have determined the energy requirements during different stages of translocation of preproteins across mitochondrial membranes using both intact mitochondria and mitoplasts. The initial translocation of the N terminus of a precursor across the inner membrane requires a membrane potential but does not require the addition of ATP or GTP. It is the subsequent translocation of the remainder of the protein which is significantly stimulated by the addition of GTP. Efficient import into the matrix, however, is achieved through coordinated interplay of cis GTP- and trans ATP-dependent processes.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mitochondria and Preparation of Mitoplasts—Mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B (ATCC24657) as described (25). To obtain mitoplasts (i.e. mitochondria with disrupted outer membrane), intact mitochondria (12.5 mg/ml in HSB (20 mM Hepes-KOH, pH 7.5, 0.6 mM sorbitol, 0.1 mg/ml bovine serum albumin)) were subjected to hypotonic shock by diluting (1:5) with the same buffer lacking sorbitol. Following incubation on ice for 15 min, samples were centrifuged at 15,000 × g for 2 min at 4 °C. The mitoplast pellet was resuspended in HSB for subsequent use. A corresponding amount of the supernatant (1.5 ml) was added back in some mitoplast import assays as indicated (see Fig. 3D).

**Expression of Precursors in Bacteria and Their Purification—** The genes encoding the precursor form of mitochondrial Hsp70 (pmt-Hsp70; Ref. 26) and Tim11 (27) were amplified from a yeast genomic library using the primers. The PCR products were subcloned into the plasmid pSP64/pCOXIV-DHFR plasmid (pET21b/pmt-Hsp70, pET21b/Tim11, or pET21b/pSDH) were cultured in M9 medium supplemented with 0.1 mg/ml ampicillin at 37 °C. When cultured to an A600 of 0.8, expression of the precursor protein was induced by the addition of isopropyl-2-thiogalactopyranoside to 1 mM. Following incubation for 15 min at 37 °C, rifampicin (0.2 mg/ml) was added to inhibit transcription by the host RNA polymerase. After another 15 min incubation at 37 °C, a mixture of [γ-32P]ATP and [α-32P]GTP was added to the reaction mixture to obtain a specific activity of 2 Ci/mmol. NTP-uptake reactions (100 µl) were performed with 50 µg of mitochondria in the import assay buffer (i.e. HSB containing 40 mM KOAc, 10 mM Mg(OAc)2, 5 mM unlabelled Met, and 1 mM dithiothreitol) essentially as described (31). Reactions were initiated by the addition of 5 µl of radioactive NTP (5 µM, 2 Ci/mmol) and immediate mixing. Following incubation at 30 °C for different time periods (10 s to 2 min), mitochondria were sedimented (15,000 × g for 2 min at 4 °C). The resulting pellet was washed with HSB, and solubilized by 0.1 ml of 2% SDS. The total radioactivity was quantitated using the software NIH Image. The bands below mPut in several figures are likely due to partial cleavage of molecules in transit to the matrix. These lower Mr bands are not the result of incomplete digestion of nonimported molecules (22).

**NTP Uptake Assay—**[α-32P]GTP and [α-32P]ATP, both with specific activity 25 Ci/mmol, were purchased from ICN Biochemicals. Labeled NTPs were mixed with the corresponding unlabeled NTP to obtain a stock of 5 µM with a specific activity of 2 Ci/mmol. NTP-uptake reactions (100 µl) were performed with 50 µg of mitochondria in the import assay buffer (i.e. HSB containing 40 mM KOAc, 10 mM Mg(OAc)2, 5 mM unlabelled Met, and 1 mM dithiothreitol) essentially as described (31). Following incubation for 2 min at 4 °C, the reaction was stopped by the addition of a solution of 5 mM ATP and 0.1 µg/ml of radioactive NTP (5 µM, 2 Ci/mmol) and immediately mixed. Following incubation at 30 °C for different time periods (10 s to 2 min), mitochondria were sedimented (15,000 × g for 2 min at 4 °C). The resulting pellet was washed with HSB, and solubilized by 0.1 ml of 2% SDS. The total radioactivity was measured using a Beckman LS 6000 liquid scintillation counter.

**RESULTS**

GTP Is Generally Required for Efficient Import of Urea-denatured Preproteins into the Matrix—We have recently shown that GTP plays a direct and essential role during efficient import of urea-denatured pPut into the matrix (22). To test whether GTP is more generally required for efficient translocation across or into the inner membrane, we tested the nucleotide requirements for import of three other purified and urea-denatured proteins: mt-Hsp70 (26), sucinate dehydrogenase (SDH; Ref. 28), and Tim11 (27). The precursor forms of both mt-Hsp70 (pmt-Hsp70) and SDH (pSDH) contain a C-terminal signal sequence which, upon import, is removed by matrix-localized signal peptidase(s). Although mt-Hsp70 is a soluble matrix protein, SDH is peripherally attached to the matrix side of the inner membrane. On the other hand, Tim11 is an integral membrane protein of the inner membrane, and its precursor form does not appear to contain a cleavable signal sequence.

Mitochondria isolated from *S. cerevisiae* were used directly without manipulating the existing NTP levels. Import of urea-denatured pmt-Hsp70 into these mitochondria was carried out in the absence or presence of ATP or GTP for two different time periods (Fig. 4A). Samples were analyzed before and after trypsin treatment. When the membrane potential across the inner membrane was dissipated by valinomycin, no import was detected even in the presence of both ATP and GTP. There was neither any conversion of the precursor to the mature form (lane 5) nor any protection of the precursor from trypsin (lane 9). In the absence of added NTPs, a small portion of the precursor was converted to the corresponding mature form (lane 2), which was completely degraded by externally added trypsin (lane 6). This shows that endogenous mitochondrial NTPs are not enough to promote import. When ATP was added, the appearance of mature molecules was increased 2.5-fold, but they were still largely accessible to protease regardless of incubation time (compare lane 3 with 7, and lane 10 with 12). Compared with the ATP-containing samples, the appearance of mature molecules was further increased another ~2-fold in the

**Import Assay—**Import reactions, consisting of mitochondria (100 µg of protein) or an equivalent amount of mitoplasts, were performed essentially as described (22). Unless otherwise indicated, isolated mitochondria or mitoplasts were used directly without manipulating the existing NTP levels. Urea-denatured precursors (40–50 ng/µl) were diluted 50-fold in the import reactions; the final urea concentration was 0.2 M. Following incubation, the mixtures were denatured with urea (0.1–0.2 mM) for 30 min on ice. To inactivate trypsin, samples were diluted with HSB containing 5 mg/ml soybean trypsin inhibitor, 100 units/ml Trasylol, and 1 mM phenylmethylsulfonyl fluoride. Mitochondria or mitoplasts were sedimented (15,000 × g for 10 min at 4 °C) and washed with 10% trichloroacetic acid. When trypsin treatment was omitted, import reaction mixtures were directly diluted with HSB containing protein inhibitor mixtures and subsequently processed as described above. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Bands representing the precursor (p) and mature (m) proteins were quantitated using the software NIH Image. The bands below mPut in several figures are likely due to partial cleavage of molecules in transit to the matrix. These lower Mr bands are not the result of incomplete digestion of nonimported molecules (22).
presence of GTP (compare lane 3 with 4, and lane 10 with 11). More importantly, a significant portion of the total mature molecules that appeared upon incubation with GTP remained protected from trypsin (compare lane 4 with 8, and lane 11 with 13). The absolute GTP-mediated import efficiency after 6 min of incubation was ~40% (lane 13), whereas the corresponding ATP-mediated import efficiency was ~5% (lane 12). GTP hydrolysis was necessary as the import was strongly inhibited by the non-hydrolyzable (or slowly hydrolyzable) analog GTP-S in a dose-dependent manner (Fig. 1B). Likewise, the import of pSDH also required the addition of GTP (Fig. 1C), and the GTP-mediated import was inhibited by GTP-S (data not shown). On the other hand, the import of Tim11 to a protease-protected location did not require the addition of GTP or ATP, although a membrane potential across the inner membrane was necessary (Fig. 1D). We conclude that GTP hydrolysis is generally required for the efficient completion of translocation of urea-denatured precursors into the mitochondrial matrix.

Efficient Import of Native Preproteins into the Matrix Also Requires GTP—We initially used urea-denatured precursors in our import assays in order to bypass any possible requirement for external ATP. However, urea-denatured precursors do not represent physiologic substrates in intact cells. Does the efficient import of a native preprotein into the matrix require GTP? To investigate this possibility (Fig. 2A, upper panel), pPut was synthesized in reticulocyte lysate cell-free translation system. Reticulocyte lysate is usually supplemented with ATP, an ATP-generating system, and GTP to support protein synthesis. A post-ribosomal supernatant containing the newly synthesized [35S]Met-labeled native pPut was therefore dialyzed extensively to remove free NTPs, NDPs, and other small molecules. This was then tested in the mitochondrial import assay. In the absence of added NTPs, about 25–30% of total pPut was converted to mPut in a membrane potential-dependent manner (data not shown). These mPut molecules, however, were not protected from externally added trypsin (lane 2), suggesting that although the signal sequence was cleaved by the matrix-localized signal peptidase, most of the C-terminal domain remained outside the organelle. This shows that residual NTPs still present in the dialysate were not sufficient to promote completion of import. Significant import was observed only when ATP or GTP was added in the import reaction. GTP-mediated import was 1.5–2-fold greater than that observed with ATP (lanes 3 and 4). This difference, however, was not as significant as that observed with urea-denatured precursors (Fig. 1, A and C; see also Ref. 22); this issue is addressed under “Discussion.”

To further determine the nucleotide specificity of import, we took advantage of the ATP-scavenging system E. coli glycerol kinase/glycerol. Only ATP can act as a phosphate donor substrate for this enzyme, which converts glycerol to glycerol-3-phosphate in a virtually irreversible manner. This system thus serves as an external ATP trap, which selectively depletes extramitochondrial ATP while sparing other NTPs (2, 22). Mi-
GTP-mediated Efficient Protein Import into Mitochondria

Figure 2. GTP plays a direct role in import of native preproteins into the matrix. A, upper panel, mitochondria were preincubated for 15 min on ice in the absence or presence of glycerol kinase (GK; 2.8 units/ml, 1.4 units/mg mitochondrial protein). All samples contained 10 mM glycerol. A post-ribosomal supernatant of reticulocyte lysate containing newly synthesized and radiolabeled pPut was extensively dialyzed and used in the import assay. Import reaction mixtures were supplemented with ATP (1 mM) or GTP (1 mM) where indicated, and incubated at 30 °C for 10 min. Samples were subjected to treatment with 0.2 mg/ml trypsin and analyzed. Lane 1 shows 10% of the pPut used per import assay. Lower panel, exactly the same as in the upper panel except pPut was replaced by native pCOXIV-DHFR synthesized in reticulocyte lysate. Lane 1 shows 20% of the pCOXIV-DHFR used in the assay. B, mitochondria were preincubated in the absence or presence of CAT (100 µg/ml) for 5 min on ice and reisolated. Mitochondria were then preincubated for 10 min on ice in the absence or presence of glycerol kinase (GK; 2.8 units/ml, 1.4 units/mg mitochondrial protein). All samples contained 10 mM glycerol. Import of dialyzed native pPut was performed in the presence of GTP (1 mM) at 30 °C for 10 min. Samples were analyzed after treatment with 0.2 mg/ml trypsin. Lane 1 shows 10% of the pPut used per import assay. (Identical results were obtained when additional CAT (100 µg/ml) was included during the import reaction as well.)

Mitochondria were pretreated with glycerol kinase, and the ATP trap was present during import as well. Under these conditions, ATP-mediated import of native pPut was completely abolished, as expected (Fig. 2A, upper panel, compare lanes 3 and 6). GTP-mediated import, on the other hand, was unaffected (lanes 4 and 7). The absolute GTP-mediated import efficiency in the absence or presence of the ATP trap was ~55% in either case. No significant decrease in import efficiency was observed when mitochondria as well as native pPut were pre-treated with glycerol kinase prior to import (data not shown). We conclude that, as in the case of urea-denatured precursors, GTP plays a direct role in the import of native preproteins into the mitochondrial matrix.

It is not known whether pPut synthesized in reticulocyte lysate folds tightly or remains loosely folded prior to import. In contrast, it has been established that fusion proteins containing a DHFR moiety fold stably before they are presented to the mitochondrial surface. They must be at least partially unfolded prior to import. In our experiments, the amount of mPut was 2–3-fold greater in the presence of ATP. When pCOXIV-DHFR was synthesized in reticulocyte lysate and subsequently treated with trypsin in the absence of mitochondria, a fragment slightly (~1–2 kDa) shorter than the DHFR moiety was generated, which remained resistant to further degradation (data not shown). This is consistent with earlier reports and confirmed that the DHFR moiety of the native fusion protein was stably folded (9).

We then tested the nucleotide requirements for import of stably folded native pCOXIV-DHFR under identical conditions used for native pPut import. As was the case for pPut, the GTP-mediated import of pCOXIV-DHFR was 1.5–2-fold greater than that observed with ATP, and was not affected by an ATP trap (Fig. 2A, lower panel). These results suggest that GTP plays a critical role also for import of preproteins with tightly folded domains.

The experiments described above (Fig. 2A) do not rule out the possibility that in the presence of an ATP trap, GTP could still lead to the generation of ATP in the IMS. This ATP could rapidly enter into the matrix via the ADP/ATP carrier, thereby escaping the external trap and stimulating import from the matrix side. This seems unlikely as the GTP-mediated pPut import remained unaffected when the ADP/ATP carrier was blocked with carboxyatractyloside (CAT; Ref. 35), whether in the absence or presence of an external ATP trap (Fig. 2B). These results further substantiate our earlier conclusion that GTP need not be converted to ATP to exert its stimulatory effect on import (22).

Role of GTP in the Translocation of a Preprotein across the Inner Membrane into the Matrix—Protein import across the outer and inner membranes of mitochondria into the matrix is a multistep process. GTP may regulate recognition and subsequent unfolding of precursors at the mitochondrial surface and/or provide energy for transmembrane movement of precursors into the organelle (22). The mitochondrial inner membrane contains a separate translocation system that is not permanently linked to that of the outer membrane (23, 24). We therefore tested the nucleotide requirements for import directly across the inner membrane into the matrix of mitoplasts.

Mitochondria were subjected to hypotonic shock, and the resulting mitoplasts were separated from soluble constituents of the IMS by centrifugation. More than 80% of endogenous cytochrome c peroxidase (IMS marker), but practically no endogenous Put (matrix marker), was detected in the supernatant (data not shown). These results indicate that hypotonic shock disrupted only the outer membrane, leaving most of the inner membrane intact. Import of native pPut (i.e. pPut synthesized in reticulocyte lysate and subsequently dialyzed) into mitoplasts was tested in the absence or presence of an ATP trap with or without addition of ATP or GTP (Fig. 3A). When no NTP was added, a significant portion of pPut was converted to mPut (lane 2). This conversion was unaffected by the presence of an ATP trap (compare lanes 2 and 8) but was completely abolished in the presence of valinomycin (data not shown). Furthermore, mPut molecules, which were generated in the absence of added NTPs, were completely degraded by externally added trypsin (lanes 5 and 11). These results suggest that the initiation of translocation across the inner membrane requires a membrane potential but does not require the addition of NTPs. When ATP was added, generation of mPut was increased 2-fold compared with a no NTP control (compare lanes 2 and 3). Still, only a very small fraction of mPut molecules was protected from trypsin (lane 6). As expected, the level of mPut production in the presence of both ATP and the ATP trap was comparable to that in the no NTP control (lanes 8 and 9), and this mPut was not protected from trypsin (lane 12). In contrast, the amount of mPut was 2–3-fold greater in the presence of
GTP than with no added NTP, regardless of the presence of the ATP trap (compare lane 2 with 4, and lane 8 with 10). More importantly, the majority of mPut molecules that were generated in the presence of GTP remained protected from trypsin (lanes 4 and 7), and the presence of the ATP trap did not inhibit the GTP-mediated completion of import (compare lanes 7 and 13). The absolute GTP-mediated import efficiency in the absence or presence of the ATP trap was ~55–60% (lanes 7 and 13). These results suggest that GTP plays a critical role during import of native pPut across the inner membrane.

A wide variety of assay conditions have been described in the literature (2, 9, 23, 24, 36–38) for protein import into mitoplasts (or mitochondria). Many import reaction mixtures contained combinations of a number of components as source of energy. In some cases, these components include ATP and an ATP-generating system because of the possibility that ATP could be rapidly hydrolyzed before it is utilized for import reactions. Other components used as source of energy are respiratory substrates (like α-ketoglutarate, succinate, or malate) and NADH to generate high matrix ATP and high membrane potential (35). In addition, some assays contained GTP. The relative contribution of each of these various components in the import process, however, remains unclear. We therefore directly compared the GTP-mediated import of urea-denatured pPut to that obtained using components other than GTP as an energy source (Fig. 3B). These assays were performed with mitoplasts without prior manipulation of endogenous NTPs. No protease-protected mature molecules were observed when the import reactions were carried out in the absence of added NTPs (data not shown). The addition of ATP alone (lane 3) or α-KG alone (lane 4) or α-KG plus NADH (lane 5) failed to stimulate import. An efficient import was observed when a combination of ATP, α-KG, and NADH (lane 6) or a combination of high concentration of ATP (2–5 mM), an ATP-generating system and NADH (data not shown) was included in the assay mixture. In contrast, GTP alone was able to drive import as efficiently as observed with ATP plus α-KG plus NADH (compare lanes 2 and 6). The GTP-mediated import was only slightly further stimulated by the combined addition of ATP, α-KG, and NADH (compare lanes 2 and 8). More importantly, the import stimulatory activity by ATP, α-KG, and NADH combined was completely abolished by the presence of an ATP trap (lane 9). On the other hand, the ATP trap failed to inhibit the import stimulatory effect mediated by a combination of GTP, α-KG, and NADH (lane 10). These results further

**Fig. 3.** GTP is required for efficient translocation of native and urea-denatured preproteins directly across the inner membrane into the matrix of isolated mitoplasts. A, where indicated, mitoplasts were pretreated with glycerol kinase (GK; 2.8 units/ml, 1.4 units/mg mitochondrial protein) and glycerol (10 mM) for 15 min on ice. Import of dialyzed native pPut was performed in the absence or presence of ATP (1 mM) or GTP (1 mM) at 30 °C for 10 min. Samples were analyzed either directly or after treatment with 0.2 mg/ml trypsin. B, where indicated, mitoplasts were pretreated with the ATP trap (glycerol kinase/glycerol) as in A. Import of urea-denatured pPut was carried out at 30 °C for 10 min in the presence of various energy sources. The final concentrations were: 1 mM GTP, 1 mM ATP, 5 mM αKG, 2 mM NADH. Samples were analyzed after treatment with 0.1 mg/ml trypsin. C, import of urea-denatured pPut was carried out at 30 °C for 20 min in the presence of ATP or GTP using either intact mitochondria (Mito, lanes 2–5) or mitoplasts (Mp, lanes 6–9). Samples were subjected to trypsin treatment (0.1 mg/ml) and analyzed. Quantitation of absolute import efficiency is presented below the autoradiograph. D, where indicated, mitoplasts were preincubated with GTP·S for 5 min on ice. ATP- or GTP-mediated import of urea-denatured pPut into mitoplasts was tested in the absence or presence of a corresponding amount of the IMS fraction. The final concentration of ATP or GTP or GTP·S was 1 mM. Following import at 30 °C for 10 min, samples were treated with trypsin (0.1 mg/ml) and analyzed.
substantiate our conclusion that GTP is the preferred source of energy.

How do we explain efficient import mediated by a combination of high matrix and external ATP (i.e. by a combination of αKG, NADH and ADP)? When matrix ATP is high, most of the externally added ATP is likely to remain outside. It is possible that a portion of this external ATP is converted to GTP. This could occur even in mitoplasts with the aid of residual IMS. GTP, so generated, together with high matrix ATP mediate an efficient import. If this is the case, it explains why externally added ATP mediates efficient import into mitoplasts only when it is combined with a respiratory substrate and NADH (Fig. 3B) or with an ATP-generating system and NADH (data not shown). Furthermore, this also explains why import mediated by a combination of αKG, NADH, and ATP is only slightly further stimulated by GTP. We cannot, however, rule out the possibility that a combination of high matrix and external ATP can bypass the GTP requirement for efficient import. The issue will be further addressed in experiments described later.

We also tested the import of urea-denatured pPut into mitoplasts and compared it with intact mitochondria (Fig. 3C). When incubated at 30 °C for a relatively long period (20 min), urea-denatured pPut was imported into intact mitochondria in the presence of ATP or GTP with comparable efficiency (lanes 2–5), in agreement with our earlier report (22). ATP-mediated import into mitoplasts under identical conditions, however, was severely diminished (lanes 6 and 7). In contrast, GTP-mediated import into mitoplasts was almost as efficient as that into intact mitochondria (compare lanes 4 and 5 with 8 and 9, respectively), and was inhibited by GTPγS (data not shown).

Depending on the conditions, we observed some ATP-mediated import of native or urea-denatured pPut into intact mitochondria (Figs. 2 and 3C), and yet ATP almost completely failed to drive import into mitoplasts (Fig. 3, A–C). The difference could be due to the existence of enzymes and/or factors in the IMS, which can slowly convert ATP to GTP. Consistent with such a mechanism, when the IMS fraction was added back in the mitoplast import assay, ATP-mediated import was stimulated by 5-fold (Fig. 3D, lanes 2 and 4). In contrast, GTP-mediated import was not stimulated to any significant extent by the addition of the IMS fraction (lanes 3 and 5). Furthermore, stimulation by the IMS fraction of ATP-mediated import into mitoplasts was strongly inhibited by GTPγS (compare lanes 4 and 6). It remains to be determined whether, in order to promote import, ATP was in fact converted to GTP with the aid of the IMS fraction. Nevertheless, ATP together with the IMS fraction clearly cannot circumvent processes that require GTP hydrolysis for import.

Effects of Blocking the ADP/ATP Carrier on NTP-mediated Import—The complex issue of the interconversion of nucleotides, presumed to occur through the action of an IMS-localized NDP kinase, perhaps could be definitively resolved by using a specific inhibitor of the enzyme. To our knowledge, no such inhibitor is known. A gene encoding the enzyme has been isolated from the yeast S. cerevisiae. The enzyme is not essential for cell viability, and the yeast strains with the disrupted gene still possess some NDP kinase activity. Furthermore, the localization of the cloned gene product is not clear (39). Likewise, no specific GTPase is commercially available. This makes it difficult to directly study the conversion of ATP to GTP during import. We therefore devised an alternative approach. Externally added ATP can enter into the matrix via the ADP/ATP carrier. If ATP is converted to GTP in the IMS compartment, one would expect that when the ADP/ATP carrier is blocked more of the added ATP will be available to be converted to GTP in the IMS, thereby stimulating import. We therefore tested the import of urea-denatured mt-Hsp70 with different concentrations of GTP or ATP when the ADP/ATP carrier was blocked with CAT. As expected, blockade of the ADP/ATP carrier had no significant effect on GTP-mediated import (Fig. 4A). In contrast, ATP-mediated import was stimulated by 2–6-fold (Fig. 4B). Similar results were obtained for the import of urea-denatured pPut (data not shown). The CAT-conferred stimulation of ATP-mediated pPut import was inhibited in a dose-dependent manner by GTPγS (Fig. 4C). These results suggest that when the external concentration of added ATP is kept high through the use of CAT, a portion of it is converted to GTP, which then stimulates import.

Comparison of the Inhibitory Effects of GTPγS with That of GDP on GTP-mediated Import—Excess GDP could interfere with the GDP/GTP exchange process of a GTPase cycle (40), thereby affecting the GTP-mediated import process. To examine this possibility, the GTP-mediated import of native pPut into mitoplasts was measured in the presence of increasing concentrations of GDP or GTPγS. Import reactions were carried out for 6 min at 30 °C (Fig. 5A). In the absence of GTP, no import was detected with GTPγS (lane 8) or GDP (lane 9) alone. As expected, GTPγS inhibited GTP-mediated import in a dose-dependent manner (lanes 3 and 4). However, under identical

![Fig. 4. Effects of blocking the ADP/ATP carrier on NTP-mediated import. A, where indicated, mitochondria were preincubated with 100 μg/ml CAT for 5 min on ice. Reaction mixtures were then supplemented with increasing concentrations of GTP. Urea-denatured pmt-Hsp70 was then added and import was carried out at 30 °C for 6 min. Samples were analyzed after treatment with 0.1 mg/ml trypsin. B, exactly the same as in A except GTP was replaced by ATP. The experiments for panels A and B were done simultaneously, and the data are from the same autoradiograph. C, mitochondria were preincubated with CAT (100 μg/ml) alone or together with GTPγS (0.25–1 μM) or both for 5 min on ice. Samples were supplemented with ATP (0.25 μM), and import of urea-denatured pPut was then carried out for 3 min at 30 °C. Reaction mixtures were treated with trypsin (0.1 mg/ml) and analyzed. The relative import efficiency was plotted against the concentration of GTPγS; the import efficiency in the absence of GTPγS was arbitrarily defined as 100%.](image-url)
The presence of GDP or GTP in the kinetics of GTP-mediated import in the absence or at time points early in the import reaction. We therefore examined a stronger GDP-mediated inhibition should be observable if bound to the active site of a putative GTPase(s) was exchanged. Somewhat surprising. One possible explanation was that GDP could be contaminated with GDP by as much as 10%. GDP associated with commercially available GTP was arbitrarily defined as 100%.

The failure of GDP to inhibit GTP-mediated import was specific and not due to any possible contamination of GDP associated with commercially available GTP-S. This is an important consideration as commercially available GTP-S (0.5 mM) or GDP (0.5 mM) for 10 min on ice. GDP (0.1 mM) and urea-denatured pPut were then added. Import was carried out at 30 °C for various time periods. Samples were treated with 0.1 mg/ml trypsin and analyzed. Lane 1 shows 5% of the pPut used per import assay. The quantitative data on relative import efficiency are presented below the autoradiograph. The GTP-mediated import efficiency in the absence of GTP-S or GDP after 10 min of incubation was arbitrarily defined as 100%.

conditions GDP failed to exhibit any inhibitory effect (lanes 5–7). These results suggest that the inhibition of import by GTP-S is specific and not due to any possible contamination of GDP associated with commercially available GTP-S. This is an important consideration as commercially available GTP-S could be contaminated with GDP by as much as 10%.

The failure of GDP to inhibit GTP-mediated import was somewhat surprising. One possible explanation was that GDP bound to the active site of a putative GTPase(s) was exchanged for GTP early in the 6-min import incubation. If this were the case, a stronger GDP-mediated inhibition should be observable at time points early in the import reaction. We therefore examined the kinetics of GTP-mediated import in the absence or presence of GDP or GTP-S (Fig. 5B). During the first 2 min, GTP-mediated import was strongly inhibited by GDP. However, this inhibition was only transient and most of it was relieved when the import incubations were continued for an additional 3 min. In contrast, GTP-S-conferring inhibition remained ~85% 5 min into the reaction. This inhibition was also relieved to some extent over a longer incubation period. This might explain why relatively high concentrations of GTP-S (3–4-fold over externally added GTP) were normally required to achieve significant inhibition of import. The kinetic data presented here are consistent with the involvement of a putative GTPase in protein import (40).

**GTP Acts Outside the Inner Membrane to Stimulate Import**—Although the ADP/ATP carrier of mitochondrial inner membrane has been extensively studied, no mitochondrial GDP/ GTP carrier or translocator has so far been described. In fact, as in the case of the chloroplast inner envelope (41), the mitochondrial inner membrane appears to be impermeable to guanine nucleotides (42–45). This suggests that GTP stimulates import from outside the inner membrane. To validate this assumption, we examined the binding/uptake of radiolabeled GTP when incubated with isolated mitochondria under our import assay conditions. Radiolabeled ATP was used as a positive control (Fig. 6). As expected, ATP was rapidly taken up by mitochondria within 10 s. In contrast, depending on the incubation time (10 s to 2 min), the amount of radiolabeled GTP associated with mitochondria was only ~15–25% of that observed with ATP. The ATP-uptake was specific as it was strongly inhibited by CAT or ADP, but not by GDP. However, this assay could not distinguish between GTP bound to proteins outside the inner membrane and GTP actually taken up into the matrix. Our observations are consistent with the accepted notion that GTP is not readily taken up by mitochondria. Although we cannot formally exclude the possibility that over a long time period small amounts of GTP can cross the inner membrane barrier, the significant GTP-mediated import of urea-denatured precursors seen in short incubation times (1–3 min; Fig. 4A and Ref. 22) makes it reasonable to conclude that GTP added to the outside also acts on the outside of the inner membrane to stimulate import.

**Involvement of GTP in the Later Stages of Protein Import**—Earlier studies have demonstrated that an electrochemical potential is needed only for the early stages of import reactions (1). In our import assays using mitoplasts, a significant portion of native pPut was processed to mPut even in the absence of
any added NTP. Although the signal sequence was cleaved by the matrix-localized signal peptidase, most of the C terminus of these mPut molecules remained exposed outside the inner membrane (Fig. 3A). The generation of these early intermediates was strictly dependent on a membrane potential (data not shown). These intermediates therefore provided a means of examining the nucleotide requirements for completion of transmembrane movement of a polypeptide chain through the inner membrane channel into the matrix.

Mitochondria were treated with CAT to block the ADP/ATP carrier and then subjected to hypotonic shock. Isolated mitoplasts were incubated with native pPut in the absence of added NTPs to generate early stage intermediates. About 55% of pPut was converted to early stage mPut intermediates (Fig. 7, lane 2) that were completely protease sensitive (lane 3). The ability of ATP, GTP or aKG to chase these early intermediates all the way into the matrix was tested after the membrane potential was dissipated with valinomycin, which prevented any further initiation of translocation across the inner membrane. GTP was able to drive to completion import of more than 75% of the protease-sensitive mPut, as measured by its acquired protease resistance (lane 7). In contrast, neither high external ATP alone (lanes 4 and 5) nor high matrix ATP alone (lane 6) was successful in this regard. More importantly, GTP-mediated completion of import was not affected by the presence of an ATP trap (compare lanes 7 and 8). These data suggest that a GTP-dependent ‘push’ from the cis side of the inner membrane is essential for efficient completion of translocation through the inner membrane channel into the matrix.

Both External GTP and Matrix ATP Are Required for Efficient Import—To delineate the individual roles of matrix ATP and external GTP, matrix ATP was reversibly modulated as follows. Efrapeptin, a potent inhibitor of the P, moiety of mitochondrial ATPase, was used to block respiration-driven ATP synthesis. In addition, the passage of ATP and ADP across the inner membrane was blocked by CAT. A combination of efrapeptin and CAT were used to deplete free matrix ATP. When these ATP-depleted mitochondria were supplemented with aKG, matrix ATP was regenerated (21, 22, 35).

When mitochondria were preincubated with efrapeptin and CAT, the GTP-mediated import was strongly inhibited (Fig. 8, compare lanes 2 and 3). The inhibition, however, was completely relieved by the regeneration of matrix ATP (lane 4); the addition of external ATP was absolutely ineffective (lane 5). As control, matrix ATP alone, even in the absence of efrapeptin, failed to stimulate import regardless of whether the ADP/ATP carrier was blocked (lane 6) or not (data not shown, but see Fig. 3B). Matrix ATP together with NADH (to generate high membrane potential) was also completely ineffective (lane 7). Likewise, no import was detected in the presence of external ATP (lane 8). We conclude that efficient import into the matrix requires the coordinated interplay of a cis GTP-dependent push and a trans ATP-dependent ‘pull.’

**DISCUSSION**

We have demonstrated that GTP, or more specifically the hydrolysis of GTP, is generally required for efficient import of preproteins into the mitochondrial matrix. GTP plays a critical role in the import of both native and unfolded preproteins. Furthermore, we have localized the GTP participation both temporally, i.e. to a particular stage of the import process, and spatially, i.e. to a particular portion of the import machinery. The transmembrane movement of preproteins up to an early stage of translocation across the inner membrane is dependent on a mitochondrial membrane potential (ΔΨ), but does not require the addition of NTPs. The efficient completion of protein translocation across the inner membrane into the matrix is independent of ΔΨ but is dependent on a GTP-mediated push (Fig. 7). Our data imply that a membrane-bound GTPase on the cis side (outside) of the inner membrane participates in import across this membrane. Efficient import into the matrix, however, requires the coordinated interplay of a cis GTP-dependent push and a trans ATP-dependent pull.

Several observations demonstrate a direct role for GTP in mitochondrial protein import. (For all comparisons described below, import refers to the appearance of protease-protected mature molecules.) GTP-mediated import of purified, urea-denatured precursors into mitochondria or mitoplasts is 1.3–50-fold more efficient, depending on the experimental conditions, than that observed with an identical concentration of ATP (Figs. 1–4; see also Ref. 22). If the GTP-mediated stimulatory effect were dependent on a conversion of the GTP to ATP, then it is very unlikely that GTP could stimulate import to a higher level than that observed with an equivalent amount of ATP.

With preproteins synthesized in reticulocyte lysate, the difference between GTP- and ATP-mediated import is much more significant in mitoplasts (at least 8-fold, Fig. 3, A and B) than in mitochondria (1.5–2-fold, Fig. 2A). This discrepancy could arise from a reduced level of IMS components present in the mitoplast preparation. It is possible that components present...
in the IMS fraction, together with the numerous enzymes present in reticulocyte lysate, facilitate the conversion of added ATP to GTP, and thereby cause an indirect stimulation of preprotein import. This notion is supported by the observation that the ATP-mediated import into mitoplasts is stimulated by the addition of IMS fraction, and this stimulation is strongly inhibited by GTPγS (Fig. 3D).

Further evidence for the direct role of GTP in import is the persistence of its stimulatory effect in the presence of an external ATP trap (Figs. 2, 3A, and 7). Because of the rapid equilibration of NTPs across the mitochondrial outer membrane, any ATP potentially generated from GTP for action in the IMS should be consumed by this external ATP trap. The formal possibility exists that generated ATP could be utilized before its equilibration across the outer membrane, but the continued stimulatory effect of GTP on mitoplast import in the presence of the same ATP trap makes this possibility unlikely (Figs. 3A and 7).

How can we exclude the possibility that ATP, potentially generated from GTP in the IMS, avoids the ATP trap by rapidly entering the matrix via the ADP/ATP carrier, and then stimulates import from the matrix side of the inner membrane? This possibility is unlikely as GTP-mediated import remains practically unaffected when the ADP/ATP carrier is blocked with CAT, whether in the absence or presence of the ATP trap (Figs. 2B and 7). More importantly, we have demonstrated that matrix ATP alone is insufficient to stimulate import (Figs. 3B, 7, and 8).

What is the site of GTP action? Guanine nucleotides themselves appear to be unable to penetrate the mitochondrial inner membrane (42–45). Our observations support this notion by demonstrating that unlike ATP, GTP is not readily taken up by mitochondria under the conditions of our import assay (Fig. 6). It is therefore likely that GTP stimulates import from outside the inner membrane. Furthermore, the efficiency of GTP-mediated import in mitoplasts is comparable to that in intact mitochondria, suggesting that soluble constituents of the IMS are not required (Figs. 3C and D). These observations implicate the action of a membrane-bound GTPase on the cis side of the inner membrane in the stimulatory effect. Support for this conclusion can be indirectly gleaned from the failure of a respiratory substrate (αKG) alone to stimulate import (Figs. 3B, 7, and 8). In the presence of αKG, substrate-level phosphorylation results in the generation of matrix ATP via the tricarboxylic acid cycle. During this process, GTP is also generated in the matrix as an intermediate (35). If GTP were to act from the matrix side of the inner membrane, αKG alone should stimulate import to some extent.

A wide variety of conditions for protein import into mitochondria or mitoplasts have been reported in the literature. These conditions vary with respect to the nature and combination of components used as the source of energy. Most import reaction mixtures, however, contained ATP, an ATP-generating system, and respiratory substrates and NADH to generate high matrix ATP and high membrane potential. Some, but not all, also contained GTP. How efficient then is the GTP-mediated import compared with that achieved under other widely used assay conditions? In agreement with earlier reports, we have also observed efficient import when both the matrix and external ATP concentrations are high, i.e. in the presence of ATP, αKG, and NADH (Fig. 3B). GTP alone, however, is able to drive import with comparable efficiency, and the GTP-mediated import is only slightly further stimulated by the combined addition of ATP, αKG, and NADH. More importantly, the presence of an external ATP trap completely abolishes the stimulation of import mediated by ATP, αKG, and NADH combined, while having no effect on the stimulation mediated by GTP alone (Fig. 3A) or a combination of GTP, αKG, and NADH (Fig. 3B). These data do not contradict those previously obtained with widely used assay systems, but rather pinpoint the relative contribution of various components normally used as energy source for mitochondrial protein import.

The individual roles of GTP and ATP are more clearly defined when the ATP concentrations both outside and inside the organelle are better controlled, i.e. when the ADP/ATP carrier is inhibited with CAT and the F$_1$ moiety of mitochondrial ATPase is inhibited with efrapeptin to block respiration-driven ATP synthesis. In the presence of both efrapeptin and CAT, GTP-mediated import is strongly inhibited. This inhibition, however, is completely relieved by regenerating matrix ATP, whereas the addition of external ATP has no effect (Fig. 8). These results might explain why an earlier study failed to detect GTP-mediated import into ATP-depleted mitochondria (20). From these data, it is reasonable to conclude that efficient import into the matrix requires the coordinated interplay of a cis GTP-dependent push and a trans ATP-dependent pull. We suggest that for efficient import it is necessary, but not sufficient, that matrix ATP and external GTP each meets a threshold level. With this requirement met, any further elevation in the level of either of these components will result in a proportional increase in import efficiency toward its maximum. In this way, high levels of matrix ATP can compensate for low, but still threshold levels of external GTP, and vice versa. In this scenario, isolated mitochondria or mitoplasts contain threshold amounts of matrix ATP but not of external GTP. The addition of external GTP to this system thus allows efficient preprotein import to be achieved.

How do we explain efficient import mediated by a combination of ATP, αKG, and NADH in the absence of externally added GTP? It is possible that a combination of high matrix and external ATP can bypass the GTP requirement for efficient import. Alternatively, in the presence of high concentrations of both matrix and external ATP, a portion of the external ATP may be rapidly converted to GTP in the IMS, presumably by NDP kinase. The GTP so generated meets the threshold requirement. This notion is supported by the observation that added ATP is more efficient at stimulating import when its external concentration is maintained at a higher level through the use of CAT, and that the stimulatory mechanism cannot bypass GTP-dependent processes (Fig. 4C). A high external ATP concentration may lead to a portion of it being converted to GTP even in mitoplasts, with the aid of the residual IMS fraction. This might explain why the import mediated by the combination of ATP, αKG, and NADH was only slightly further stimulated by GTP (Fig. 3B). The conversion of ATP to GTP in mitoplasts is expected to be suboptimum, and the efficient import can therefore be seen only in the presence of high concentrations of matrix ATP generated by respiratory substrate(s) (Figs. 3 and 7).

An enzymatic conversion of ATP to GTP is expected to be temperature-dependent. Such an exchange process may be apparent in the kinetics of import at different temperatures. We have previously shown that the GTP-mediated import of ureadenatured pPut can occur to a significant extent even at lower temperatures (0–10 °C). In contrast, ATP can drive import to levels above 5% only at 30 °C. After a short incubation at 30 °C, GTP-mediated import reaches a plateau level, whereas ATP-mediated import is still in the early stage of the ascending linear range (22). With longer incubation (~20 min) at 30 °C, the difference between GTP- and ATP-mediated import is diminished (Fig. 3C). These results provide a possible explanation as to why some earlier studies, where import incubations

GTP-mediated Efficient Protein Import into Mitochondria

20949
were performed for longer durations at higher temperatures, failed to detect the GTP stimulatory effect over that mediated by ATP (22).

How does GTP stimulate mitochondrial protein import? We propose that the completion of protein translocation through the inner membrane channel into the matrix requires participation of a membrane-bound GTPase on the cis side of the inner membrane. The GTPase may transiently accompany the polypeptide chain into the inner membrane channel, as in the case of ATP-powered push by SecA in protein transport across the bacterial plasma membrane (for review, see Refs. 46 and 47). Alternatively, the GTPase may feed segments of the polypeptide chain into the inner membrane channel without actually penetrating the channel itself. The GTP-dependent push of the polypeptide chain across the inner membrane, in turn, may lead to unfolding of C-terminal domains that are yet to enter the organelle. The GTP requirement for efficient import, however, cannot be circumvented by altering the structural integrity of preproteins and/or by presenting them directly to the inner membrane in order to eliminate the need for translocation across the outer membrane.

An efficient unidirectional transmembrane movement of proteins across the inner membrane into the matrix is achieved only through the coordination of cis GTP- and trans ATP-dependent processes. Both of these processes are necessary; neither one can substitute for the other. The GTP-dependent push might be disengaged after mt-Hsp70 has sufficiently grabbed the incoming polypeptide chain. Alternatively, the process might continue until import is complete. The precise role of GTP in mitochondrial protein import can be determined only after identification of a relevant GTPase(s).

GTP, shown here to be required for efficient import of proteins across the inner membrane into the matrix, may also participate in other stages of mitochondrial import, such as protein targeting. Indeed, GTP plays an important role in regulating protein targeting to the endoplasmic reticulum and chloroplasts (for review, see Refs. 48 and 49). Furthermore, as in the case of mitochondria, the outer and inner envelope membranes of chloroplasts contain independent protein translocation systems (50), protein import into chloroplast stroma requires stromal ATP (51), and the inner envelope membrane is impermeable to GTP (41). In light of the data presented here, it would be worthwhile to determine whether a similar GTP-dependent push and ATP-dependent pull is necessary for translocation of preproteins across the inner envelope membrane into the chloroplast stroma.

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