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tRNA-triggered ATP Hydrolysis and Generation of Membrane Potential by the *Leishmania* Mitochondrial tRNA Import Complex*

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Translocation of tRNAs across mitochondrial membranes is a receptor-mediated active transport process requiring ATP. A large tRNA import complex from the inner membrane of *Leishmania* mitochondria catalyzes translocation into phospholipid vesicles. In this reconstituted system, the import substrate $tRNA^{Tyr}$(GUA) specifically stimulated hydrolysis of ATP within the vesicles, with the subsequent generation of a membrane potential by pumping out of protons, as shown by the protonophore-sensitive uptake of the potential-sensitive dye rhodamine 123. Generation of membrane potential was dependent on ATP hydrolysis, and inhibited by oligomycin, recalling the proton-translocation mechanism of the respiratory F_1-F_0-ATPase. For translocation of tRNA, ATP could be replaced by low pH of the medium, but proton-dependent import was resistant to oligomycin. Moreover, ATP hydrolysis, generation of membrane potential and tRNA uptake were inhibited by carboxyatractyloside, a specific inhibitor of mitochondrial ATP-ADP translocase, implying an ATP requirement within the vesicles. These observations imply a gating mechanism in which tRNA, on binding to its receptor, triggers the energetic activation of the complex, leading to the opening of import channels.

In many species across the phylogenetic scale, the mitochondrial genome is incomplete with respect to the number of tRNA genes required for translation of the organellar mRNAs (1–6). In such cases, mitochondrial translation is sustained by the import of nucleus-encoded tRNAs from the cytoplasm. The mechanism of translocation of these highly negatively charged macromolecules across the hydrophobic and electrostatic barriers of two mitochondrial membranes (of which the inner membrane is negatively charged in respiring mitochondria due to selective proton expulsion from the matrix) is a matter of considerable interest but poorly understood. Particularly, the bioenergetic inputs required to overcome these barriers have not been precisely defined.

Using *in vitro* systems, it was shown that tRNA import in yeast, *Leishmania* and *Trypanosoma* mitochondria is ATP-dependent (7–10) and requires ATP hydrolysis (11). ATP could have a direct role in inducing conformational changes leading to opening of import channels. Alternatively, the mitochondrial F_1-F_0-ATPase, or some other ATPase dedicated to import, could generate a proton gradient that actually drives import. A role of protons was indicated by the inhibitory effect of protonophores (11), which dissipate proton gradients, but whether protons are sufficient to induce import remains an open question. Finally, it is unclear whether proton gradient generation and tRNA translocation are separate or coupled processes and whether tRNA itself plays an active role in proton translocation.

We have recently reported the isolation of a multiprotein complex (the RNA import complex or RIC)[1] that is sufficient to induce import of tRNAs into artificial phospholipid vesicles (12). This reconstituted system retains all the properties of import in intact mitoplasts, including ATP dependence and sensitivity to respiratory uncouplers and inhibitors (12). Two tRNA-binding proteins were identified within this complex by photo-cross-linking and immunochemistry, a 45-kDa protein that binds $tRNA^{Tyr}$ directly and another 21-kDa protein that binds $tRNA^{Ile}$ only in the presence of $tRNA^{Tyr}$, suggesting allosteric changes within RIC leading to modulation of tRNA affinities (12–14). In the present study, we have used the RIC-reconstituted liposome system to study the bioenergetic transactions leading to import.

MATERIALS AND METHODS

Isolation of Mitochondria and Purificaton of RIC—Mitochondia were prepared from *Leishmania tropica* strain UR6 and purified by Percoll gradient centrifugation (8). Mitoplasts were prepared by digitonin treatment as described previously (11). RIC was purified from mitochondrial inner membranes as described (12). Briefly, mitochondrial inner membranes were extracted with 0.5% sodium deoxycholate in presence of 0.4 M KCl, and the extract was subjected to affinity chromatography using as ligand, the D arm of $tRNA^{Tyr}$, which contains an import signal (14, 16). The affinity-purified fraction was used for ATPase activity assays or for proteoliposome reconstitution.

Preparation of Proteoliposomes—Empty liposomes were prepared (12) by suspending phosphatidyl choline (250 μg) in 50 mM HEPES-KOH, pH 7.5, 10 mM $MgAc_2$, 2 mM DTT, 10% glycerol, 0.25% deoxycholate and dialyzing the suspension against 5 mM HEPES-KOH, pH 7.5, 5 mM $MgAc_2$, 20 mM KCl for 18 h at 4 °C. Aggregated material was removed by centrifugation at 2300 × *g* and the supernatant, containing mainly unilamellar vesicles, was used. For reconstitution, liposomes (25 μg) and RIC (100 ng) were incubated in the same buffer without deoxycholate for 1 h before import or ATPase assays.

Import Substrates—^{32}P-Labeled $tRNA^{Tyr}$(GUA) or $tRNA^{Gln}$(CUG) was prepared by T7 RNA polymerase transcription as described previously (15).

Import Assays—Proteoliposomes (equivalent to 100 ng of RIC) were incubated with 100 fmol of substrate RNA in 20 μl of import buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 4 mM ATP) at 37 °C for 15 min, then RNases A and T1 were added at final concentrations of

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[1] The abbreviations used are: RIC, RNA import complex; DTT, dithiothreitol; PEI, polyethyleneimine; TLC, thin layer chromatography; STE, sucrose-Tris-EDTA; ANT, adenine nucleotide translocator; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

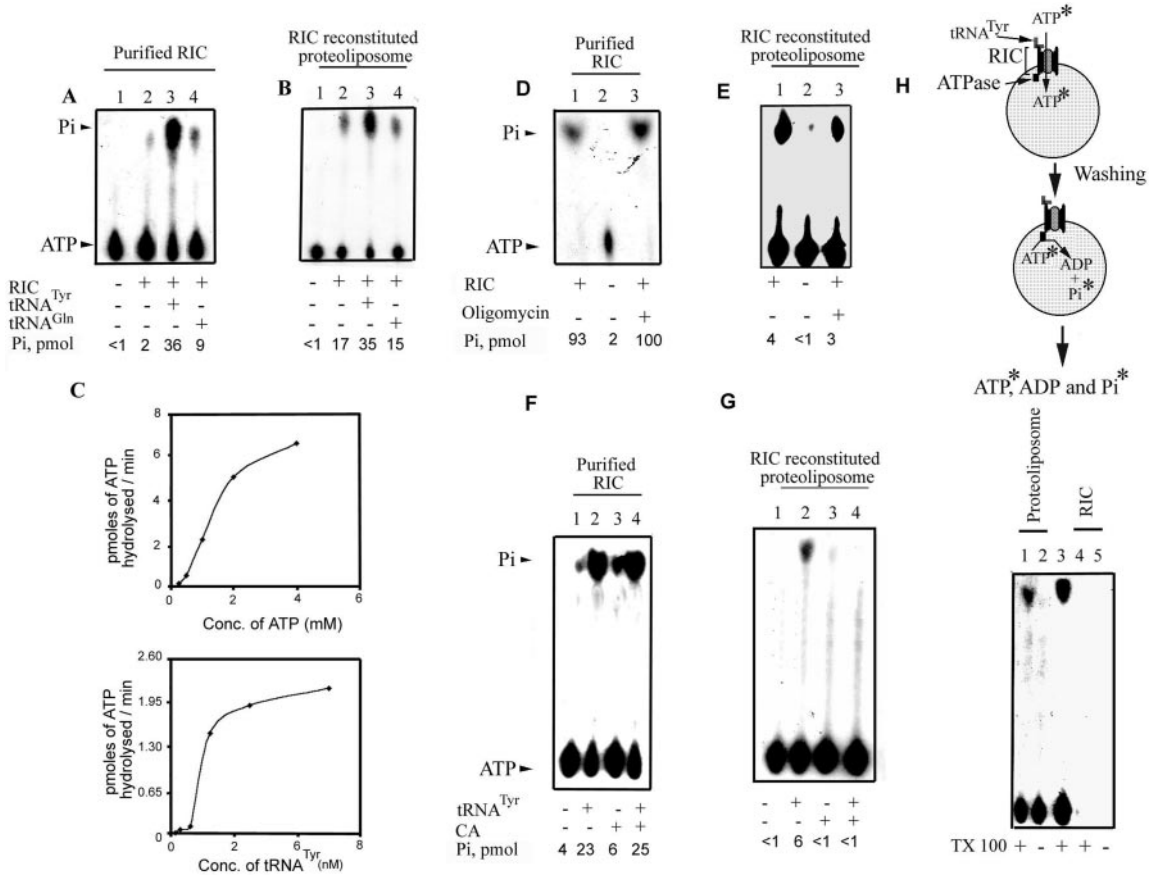

FIG. 1. **tRNA-stimulated ATPase activity of RIC.** *A*, ATPase activity in the absence (*lane 1*) or presence (*lanes 2–4*) of soluble RIC (10 ng) or in the absence (*lane 2*) or presence of 5 nM $tRNA^{Tyr}$ (*lane 3*) or $tRNA^{Gln}$ (*lane 4*). An aliquot of each reaction was analyzed by thin layer chromatography on PEI-cellulose. *B*, as in *A*, except that reconstituted RIC-liposomes (from 10 ng of RIC) instead of the soluble complex were used. *C*, rate of ATP hydrolysis by soluble RIC (2 μg/ml) as a function of ATP concentration (*Conc.*) (*upper panel*) or $tRNA^{Tyr}$ concentration (*lower panel*). *D*, $tRNA^{Tyr}$-dependent soluble RIC-ATPase in the absence (*lane 1*) or presence (*lane 3*) of 50 μM oligomycin. *E*, effect of oligomycin (50 μM) on ATPase activity of RIC-liposomes. Additions were as described in the legend to *D*. *F* and *G*, effect of carboxyatractyloside (*CA*, 50 μM) on tRNA-stimulated ATPase activity of soluble (*F*) or liposomal (*G*) RIC. *H*, the site of ATP hydrolysis in RIC-liposomes. *Top*, experimental protocol. After incubating in presence of $tRNA^{Tyr}$ and [γ-^{32}P]ATP, RIC-liposomes were suspended in STE and separated from external ATP by ultrafiltration. *Bottom*, liposomes retained by the filter were resuspended and divided into two aliquots, one of which was treated with 0.5% Triton X-100 (*TX 100*). Both treated (*lane 1*) and untreated (*lane 2*) aliquots were analyzed by TLC. In *lanes 3–5*, reaction products of soluble RIC before (*lane 3*) or after ultrafiltration (*lanes 4* and *5*) were analyzed.

2.5 μg/ml and 50 units/ml, respectively, and RNase digestion continued at 37 °C for 15 min. The proteoliposomes were then washed with 3 ml of liposome washing buffer (5 mM HEPES-KOH, pH 7.5, 2 mM DTT, 5 mM EDTA) and the vesicles recovered by centrifugation at 120,000 × *g* for 30 min at 4 °C. RNA was isolated from the liposomes as described previously (12, 16).

ATPase Assay—RIC (10 ng) or proteoliposomes reconstituted with 10 ng of RIC (25 μg of phopholipid) was incubated in a 5-μl reaction with 1 mM [γ-^{32}P]ATP (specific activity 2000 cpm/pmol) in 10 mM Tris-HCl, pH 8.0, 10 mM $MgAc_2$, and 2 mM DTT, in the presence or absence of low specific activity (5000 cpm/pmol) tRNA (5 nM) as indicated. Inhibitors were preincubated with RIC or proteoliposomes on ice for 15 min prior to addition of the assay mix. After incubation at 37 °C for 10 min, 1 μl of each reaction was mixed with 1 μl of 1% SDS, deoxycholate, or Triton X-100 and spotted on prewashed polyethyleneimine (PEI) cellulose thin layer chromatography (TLC) plates (Sigma), essentially as described previously (17). The plates were developed by sequential runs in 0.1 M acetic acid and 1 M LiCl and autoradiographed. Radioactive spots were quantified by liquid scintillation counting or densitometry. Apparent K_m values were estimated from rate curves as the concentration of ATP or $tRNA^{Tyr}$ for half-maximal activity (pmol of ATP hydrolyzed per min). To determine the site of ATP hydrolysis, proteoliposomes were washed with isotonic sucrose-Tris-EDTA (STE; Ref. 8) and separated from free, *i.e.* external ATP by centrifugal ultrafiltration with a Microcon 30 ultrafilter (Amicon) at 16,000 × *g*, and resuspended in 10 μl of STE for TLC.

Rhodamine 123 Uptake Assay—RIC-proteoliposomes (10 μg of lipid and 3 ng of RIC) were incubated with 0.05% rhodamine 123 in 10 μl of import buffer with 4 mM ATP for 15 min at 37 °C. Wherever indicated, liposomes were preincubated with inhibitors at 4 °C for 15 min. The proteoliposome suspension was mixed with an equal volume of STE containing 20% glycerol and observed under a Leica DMIRB inverted confocal microscope with laser excitation at 488 nm and emission at 540–668 nm. Images at ×1000 magnification were analyzed and quantified by the Leica SP2 confocal software. The mean fluorescence intensity per particle was calculated as the product $f_{av} \times n_f$, where f_{av} = average intensity of fluorescence in stained particles, and n_f = fraction of particles staining above background.

RESULTS

A tRNA-stimulated ATPase Activity in RIC—To determine whether the ATP-hydrolytic activity required for import is an integral part of, or distinct from, RIC, the purified complex was incubated with [γ-^{32}P]ATP and radiolabeled P_i separated by thin layer chromatography. At high concentrations of RIC, a nonspecific ATPase activity was observed (data not shown). However, at low RIC concentrations, ATP hydrolysis was stimulated ~16-fold by the import substrate $tRNA^{Tyr}$ (Fig. 1*A*). In contrast, $tRNA^{Gln}$(CUG), which is not imported *in vivo* or *in vitro*, had only a 4-fold effect (Fig. 1*A*); total *Escherichia coli* tRNA had no effect (data not shown). The preference of ATPase stimulatory activity for an importable tRNA indicates the relevance of this ATPase to import. From rate curves (Fig. 1*C*), the apparent $K_m^{tRNA(Tyr)}$ for ATP hydrolysis was 0.98 nM, which is close to the value of 1.16 nM for RIC-mediated import (12).

Similarly, K_m^{ATP} for the ATPase was 1.32 mM, compared with 1.35 mM for import (12).

To examine further the relationship between the RIC ATPase and import, the complex was incorporated into liposomes. Incubation of the proteoliposomes with ATP resulted in $tRNA^{Tyr}$-stimulated ATP hydrolysis (Fig. 1*B*), indicating retention of the activity in a membrane-bound form of the enzyme. However, the degree of stimulation of membrane-bound ATPase by $tRNA^{Tyr}$ was 2-fold compared with 16-fold for the soluble complex. This could be due to partial denaturation, with concomitant exposure of the ATPase site and loss of allosteric regulation, on insertion of the complex into the membrane.

Import both *in vitro* (11, 12) and in transfected cells (14) has been shown to be sensitive to oligomycin, a specific inhibitor of the mitochondrial F_1-F_0-ATPase. Oligomycin apparently exerts its effect by reducing the permeability of the F_0 portion of the enzyme to protons (18). The ATPase activity of RIC was resistant to oligomycin, both in the free (Fig. 1*D*) and membrane-bound (Fig. 1*E*) forms. Thus, the oligomycin-sensitive step in import must be after the initial ATP hydrolysis step.

ATP Hydrolysis Inside the Liposome—tRNA-stimulated ATP hydrolysis by membrane-bound RIC could occur on either side of the liposome membrane. The site of hydrolysis was determined in two ways. First, we had previously observed that import in intact mitochondria (11) or mitoplasts[2] is sensitive to carboxyatractyloside, a specific inhibitor of the adenine nucleotide translocator (ANT) on the inner membrane, suggesting the requirement of transport of ATP into the matrix. In the RIC-liposome system, tRNA uptake was similarly sensitive to carboxyatractyloside (Fig. 3*C*). In the same system, ATP hydrolysis was also inhibited by the drug, but the ATPase activity of the free RIC was unaffected (Fig. 1, *F* and *G*). These results are consistent with the presence of ANT, or an ANT-like carrier, in the complex and further indicate that upon insertion of RIC into the membrane the ATPase active site is exposed to the inner compartment.

In a second experiment, we directly checked for the presence of labeled ATP and P_i within the vesicles as follows. RIC-liposomes were incubated with [γ-^{32}P]ATP and separated from free ATP by ultrafiltration. When the liposomes were directly run on TLC plates, most of the radioactivity was concentrated near the origin, with little or no free P_i near the solvent front (Fig. 1*H*, *lane 2*). However, P_i was released upon treatment of the liposomes with Triton X-100 (Fig. 1*H*, *lane 1*). In a control experiment, free RIC was similarly incubated with ATP and ultrafiltered; neither ATP nor P_i was detected in the retentate (Fig. 1*H*), excluding the possibility of RIC itself binding or protecting significant amounts of these molecules. This experiment confirms the uptake and hydrolysis of ATP within the proteoliposome.

RIC- and tRNA-dependent Generation of Membrane Potential—The lipophilic cationic dye rhodamine 123 is taken up into respiring mitochondria of living cells in a membrane potential-dependent manner (19). We have previously observed uptake of rhodamine 123 into isolated *Leishmania* mitochondria in presence of ATP (11). The availability of the RIC-liposome system allowed the development of a microscopic assay of membrane potential generation by rhodamine 123 uptake.

Control or RIC-reconstituted liposomes were incubated with tRNA and ATP and uptake of rhodamine 123 monitored by confocal fluorescence microscopy. About 80% of the vesicles became labeled in the complete system (Fig. 2). Uptake was dependent on the presence of RIC, ATP, and $tRNA^{Tyr}$. ATP

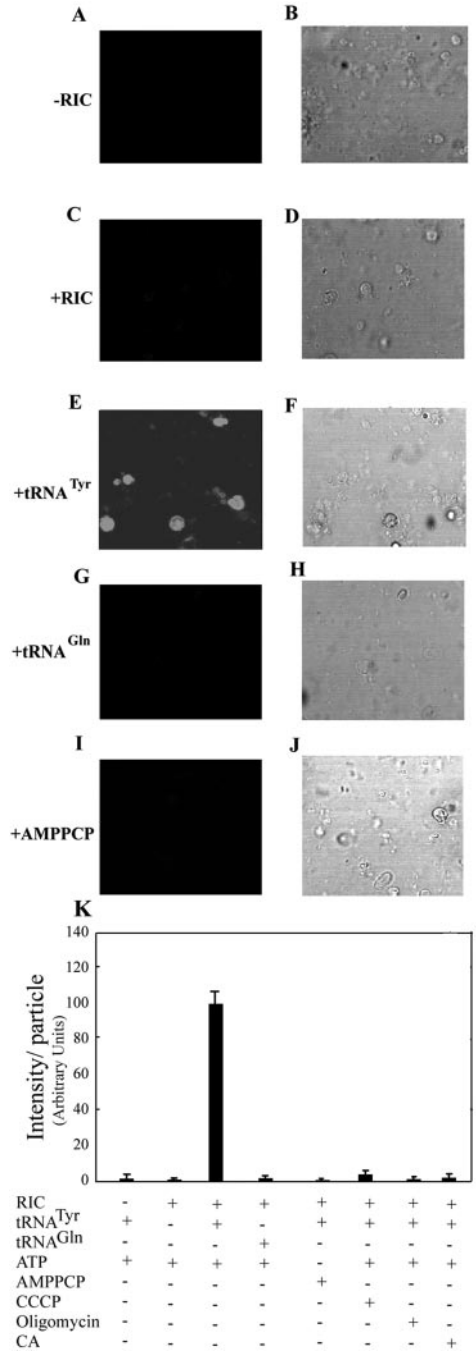

FIG. 2. **RIC- and tRNA-dependent uptake of rhodamine 123 into liposomes.** Liposomes were incubated with rhodamine 123 (0.05 μg/ml) and observed by fluorescence confocal microscopy (×1000). Fluorescence images (*A*, *C*, *E*, *G*, and *I*) and corresponding phase contrast images (*B*, *D*, *F*, *H*, and *J*) are shown. *A* and *B*, complete minus RIC. *C* and *D*, complete minus tRNA. *E* and *F*, complete system, containing liposomes (25 μg), RIC (10 ng), ATP (4 mM), and $tRNA^{Tyr}$ (5 nM). *G* and *H*, $tRNA^{Gln}$(CUG) replaced $tRNA^{Tyr}$. *I* and *J*, AMPPCP replaced ATP. *K*, average fluorescence intensity per liposome (arbitrary units) for various reactions, as indicated.

could not be substituted by the non-hydrolyzable analogue AMPPCP, showing a requirement for ATP hydrolysis. $tRNA^{Tyr}$ could not be replaced by the non-importable $tRNA^{Gln}$(CUG). Moreover, uptake was inhibited by the protonophore uncoupler *m*-chlorocarbonylcyanide phenylhydrazone (CCCP), which dissipates trans-membrane proton gradients; this implies that the membrane potential is generated by RIC-mediated pumping of protons. Uptake was also completely sensitive to oligomycin, a proton channel blocker. Inhibition of ATP transport by car-

[2] S. N. Bhattacharyya and S. Adhya, unpublished data.

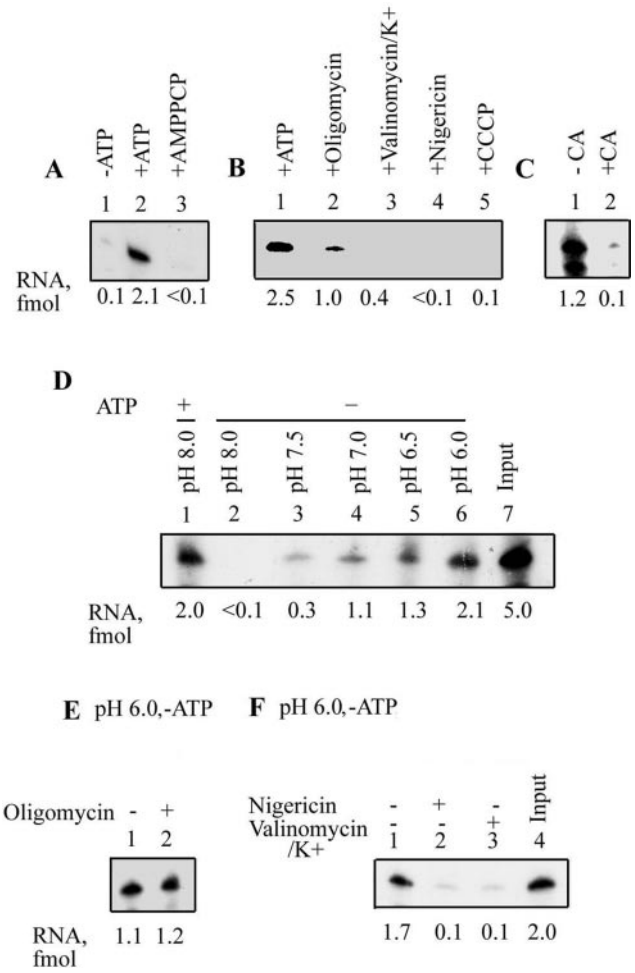

FIG. 3. **Proton-driven import in proteoliposomes.** *A*, ATP dependence. Import reactions were carried out with RIC-liposomes in the absence (*lane 1*) or presence of 4 mM ATP (*lane 2*) or of 4 mM AMPPCP (*lane 3*). *B*, effect of inhibitors. Import was assayed in the absence of inhibitor (*lane 1*) or in presence of 50 μM olgomycin (*lane 2*), 50 μM valinomycin and 100 mM KCl (*lane 3*), 50 μM nigericin (*lane 4*), or 50 μM CCCP (*lane 5*). *C*, effect of carboxyatractyloside. Import reactions were carried out in the absence (*lane 1*) or presence (*lane 2*) of 50 μM carboxyatryctyloside (*CA*). *D*, effect of pH. Import was carried out at pH 8.0 in the presence of ATP (*lane 1*) or at pH 8.0, 7.5, 7.0, 6.5, or 6.0 in the absence of ATP (*lanes 2–6*, respectively). *Lane 7*, input $tRNA^{Tyr}$, 5 fmol. *E*, effect of oligomycin (50 μM) on import at pH 6.0 in absence of ATP. *F*, effect of 50 μM nigericin (*lane 2*) or of 50 μM valinomycin plus 100 mM KCl (*lane 3*) on pH 6-driven import.

boxyatractyloside abolished uptake of rhodamine 123, implying the requirement of ATP hydrolysis within the vesicles. These results indicate the generation of a membrane potential (negative inside) by a proton pump activity of RIC.

Proton-driven Import of tRNA—ATP-dependent import of tRNA in mitochondria (11) and in transfected cells (14) has been shown to be sensitive to protonophores and K^+/H^+ ionophores that dissipate either or both of the components (electrical or chemical) of the electrochemical gradient set up by proton translocation in respiring mitochondria. In RIC-reconstituted liposomes, ATP-dependent uptake of tRNA was also sensitive to CCCP, valinomycin, and nigericin (Fig. 3, *A* and *B*). This indirectly suggests that it is the proton gradient generated by ATP hydrolysis, rather than ATP hydrolysis itself, that provides the driving force for import but does not exclude an additional role for ATP. Therefore, we checked whether ATP can be replaced by protons in the RIC-reconstituted system. Uptake assays were performed in buffers of different pH, the internal pH of the liposomes being 8. At an external pH of 8, there was no uptake in absence of ATP (Fig. 3*D*), but as the external pH was progressively lowered, tRNA uptake increased until, at an external pH of 6, it reached the value obtained with ATP. Proton-dependent uptake was similarly sensitive to valinomycin and nigericin (Fig. 3*F*). The K^+ ionophore valinomycin, in presence of high external K^+, dissipates the electrical component (the membrane potential, $\Delta\psi$) of the protonmotive force, whereas nigericin, which exchanges H^+ for K^+, disrupts the chemical component (ΔpH) without affecting $\Delta\psi$. Thus, these experiments indicate the requirement of both components for RIC-mediated import.

An interesting difference between ATP-dependent and proton-dependent import was observed with respect to their responses to oligomycin. Whereas ATP-dependent import was sensitive to oligomycin (Fig. 3*B*), proton-dependent import was resistant (Fig. 3*E*). This indicates that, in the energy-transduction pathway, the oligomycin-sensitive step occurs between ATP hydrolysis and proton-driven translocation, *i.e.* at the step of proton extrusion with concomitant generation of a membrane potential (see above).

DISCUSSION

The binding of substrate tRNA to its receptor on the RIC has multiple effects on import-related processes. It allosterically influences the binding of other tRNAs: a Type I tRNA stimulates binding of a Type II tRNA, while a Type II tRNA inhibits the binding of a Type I tRNA (12–14). It may induce the opening of the import channel (12). In addition, tRNA specifically stimulates an ATPase activity (Fig. 1) and induces a membrane potential (Fig. 2). Our current results are consistent with a multistep pathway of energy transduction within the complex leading, ultimately, to tRNA translocation; 1) substrate tRNA binds to its receptor; 2) ATP is transported into the mitochondrion via a translocase, which is similar or identical to the carboxyatractyloside-sensitive adenine nucleotide transporter; 3) tRNA-receptor binding leads to the activation of an RIC-associated ATPase which catalyzes ATP hydrolysis within the mitochondrion; 4) ATP hydrolysis is accompanied by pumping of protons through an oligomycin-sensitive channel from inside to outside the membrane, resulting in the generation of a membrane potential; 5) establishment of the proton gradient leads to opening of the import channel, possibly by voltage gating; this would explain the requirement for the membrane potential, $\Delta\psi$; 6) the tRNA is transported through the import pore. Excess external protons (ΔpH) are required at this step. The protons may act to neutralize the negative charges on the RNA phosphate groups or line the pore to allow "electrophoretic" translocation of the RNA.

The RNA-stimulated ATPase (which we designate as R-ATPase) appears to be functionally related to, but distinct from, the mitochondrial respiratory F-ATPase and the vacuolar V-ATPase (for a comparison of the latter two enzymes, see Ref. 20). In all three cases ATP hydrolysis is coupled to proton translocation across the membrane. Moreover, proton movement occurs away from the compartment in which ATP hydrolysis is occurring, and both F- and R-ATPases, in their membrane-bound form, pump protons in an oligomycin-sensitive manner. There are also structural similarities; spherical particles resembling the knobs of the F1 sector of F-ATPase have been observed with purified RIC preparations (12). Additionally, our recent peptide sequencing studies[3] reveal the occurrence of an RIC subunit with homology to the α-subunit of F-ATPase, which is also present in V-ATPase (20). Thus the F-,

[3] S. N. Bhattacharyya, S. Chatterjee, S. Goswami, and S. Adhya, unpublished data.

V-, and R-ATPases may belong to a superfamily of multiprotein complexes dedicated to different intracellular functions.

One interesting aspect of the RIC is the ease with which it induces tRNA translocation across membranes; simple addition to, or preincubation with membrane vesicles, whether liposomes (12) or mitoplasts or intact mitochondria (unpublished data), is sufficient, and exhaustive reconstitution by dialysis, as is common with respiratory complexes (21), is unnecessary. This suggests a facile insertion of the complex into membranes. Moreover, membrane insertion of the complex may be non-random. This is indicated by the fact that in the reconstituted liposome, ATP hydrolysis occurs internally, and the R-ATPase activity is totally sensitive to carboxyatractyloside (Fig. 1), whereas the soluble ATPase is resistant, implying that most or all of the ATPase active sites are internal to the membrane; random insertion would be expected to yield about 50% carboxyatractyloside-resistant ATPase activity. The mechanism of facile, non-random insertion is unknown; one possibility is insertion via a lipid anchor of the type found in *Leishmania* and *Trypanosoma* surface glycoproteins (22).

It is curious that the mitochondrial inner membrane contains a mechanism for generating a membrane potential that is independent of respiratory chain-mediated electron transport; indeed, we have observed that with intact mitochondria or mitoplasts, respiratory substrates such as succinate, citrate, etc. are unable to replace ATP as an energy source for import.[4] Thus the question arises as to whether the tRNA-dependent proton gradient generated by RIC is dedicated to import or whether it can be utilized under certain conditions to catalyze other inner membrane transport processes. The present study reveals the presence of several entities within the RIC that appear to be related to known mitochondrial components involved in ATP synthesis and transport: an oligomycin-sensitive ATPase, proton channels, and an ATP carrier. It will be important to identify the corresponding proteins to establish the relationship between tRNA import and oxidative phosphorylation.

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[4] S. N. Bhattacharyya, S. Mukherjee, and S. Adhya, unpublished data.

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