J. Membrane Biol. 110, 227–233 (1989)

The Journal of
Membrane Biology


# Solubilization and Reconstitution of the *Rickettsia prowazekii* ATP/ADP Translocase

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**Summary.** The ATP/ADP translocase protein of *Rickettsia prowazekii*, an obligate intracellular parasite that had been grown in the chick yolk sac, was solubilized and reconstituted into liposomes composed of *Escherichia coli* phospholipid by an octylglucoside dilution procedure. Proteoliposomes prepared from membranes of Renografin-purified *R. prowazekii* translocated ATP by an obligate exchange mechanism. Influx of extravesicular ATP required intravesicular transportable nucleotide and efflux of intravesicular ATP required transportable extravesicular nucleotide in the medium. The transport activity was insensitive to carboxyatractyloside and bongkrekic acid, inhibitors of mitochondrial ADP/ATP translocation. Proteoliposomes prepared from membranes of standard (non-Renografin-purified) *R. prowazekii* exhibited both an inhibitor-sensitive mitochondrial translocase activity and an inhibitor-resistant rickettsial translocase activity. Proteoliposomes prepared from uninoculated yolk sac membranes exhibited only the inhibitor-sensitive mitochondrial translocase activity. The substrate specificity of each reconstituted translocase was determined and shown to correspond with that reported for intact mitochondria or rickettsiae. Following influx of ATP the steady-state value for intravesicular labeled ATP was dependent on the concentration of intravesicular nucleotide available for exchange.

**Key Words** proteoliposomes · ATP/ADP translocase · *Rickettsia prowazekii* · reconstitution · octylglucoside · mitochondria

## Introduction

The acquisition of nutrients is a fundamental process common to all living organisms. How an individual organism satisfies its nutritional requirements is dependent upon the metabolites available and the metabolic repertoire of the organism. *Rickettsia prowazekii*, the etiologic agent of epidemic typhus, grows and multiplies only within the cytoplasm of a eucaryotic host cell. This unusual ecological niche provides the rickettsiae access to metabolites that free-living bacteria might never encounter in their environment. Understanding how rickettsiae efficiently compete with the host cell for these metabolites is fundamental to understanding both rickettsial biology and the rickettsial disease process.

An interestiang aspect of rickettsial metabolism is that *R. prowazekii* possesses an ATP/ADP exchange transport system [16]. In isolated rickettsiae the transport of ADP and ATP occurs by obligatory exchange. That is, for every ADP or ATP molecule entering the rickettsiae an ADP or ATP molecule must also be expelled. In the host cell we presume that the rickettsiae predominately exchange host cell ATP for rickettsial ADP. The net result of this exchange is the transport of a high energy phosphate bond into the rickettsial cell. The metabolic constraints which maintain this directionality are not yet fully understood. However, the transport of ATP over ADP is favored when the phosphate concentration of the surrounding medium is low [17].

Mitochondria possess an analogous system for the exchange of ADP and ATP [3]. However, the physiological directionality of ATP transport is in opposite directions for these two functionally similar transport systems. The mitochondrial translocase predominantly catalyzes the exchange transport of cytoplasmic ADP for mitochondrial matrix ATP. In addition, the mitochondrial transport system is sensitive to a number of specific inhibitors such as carboxyatractyloside [12] and bongkrekic acid [1, 2, 4]. These inhibitors have shown no effect on the rickettsial ATP/ADP transport system [16]. The *R. prowazekii* ATP/ADP translocase gene has been cloned and expressed in *E. coli* [6]. DNA sequence analysis has revealed an open reading frame of 1,494 base pairs that could encode a hydrophobic protein with a molecular weight of 56,668 and an isoelectric point (pI) of 10.3 [15]. Comparisons of the deduced amino acid sequence of the *R. prowazekii* ATP/ADP translocase with that of mitochondrial translocases revealed no detectable homologies.

In order to further characterize the rickettsial

ATP/ADP translocase protein purification and functional reconstitution of the translocase in phospholipid vesicles are necessary. Functional reconstitution of the rickettsial translocase would provide an assay for the purification of the carrier. Furthermore, reconstitution would provide a method for further elucidating the molecular mechanism of ATP/ADP exchange.

This paper describes the solubilization and reconstitution of the rickettsial ATP/ADP translocase into liposomes prepared from *E. coli* phospholipids. Reconstitution of a eucaryotic mitochondrial translocase was also documented. A comparison of the two reconstituted translocase activities is presented.

## Materials and Methods

### Rickettsial Preparation and Growth

*R. prowazekii,* Madrid E strain, was propagated in 6-day embryonated, antibiotic-free hen eggs by inoculation with a dilution of a seed pool. Infected yolk sacs were harvested 8 days postinoculation, and rickettsial suspensions were prepared from heavily infected yolk sacs and further purified at 4°C as previously described [16]. Uninoculated yolk sac preparations were prepared by a similar procedure, except that the celite, albumin and $MgCl_2$ steps were not included. Renografin density gradient purification, when used, was done by a variation of the method of Weiss et al. [13]. The rickettsiae were layered onto 25% Renografin (Squibb, Princeton, NJ) using SPG (218 mM sucrose, 3.76 mM $KH_2PO_4$, 7.1 mM $K_2HPO_4$, and 5 mM glutamic acid, pH 7.0) as the diluent. Centrifugation was carried out at 15,000 rpm for 30 min at 4°C in an SA-600 rotor (Dupont Sorvall, Norwalk, CT). Rickettsiae sedimented in this manner are referred to herein as Renografin-purified rickettsiae.

### Membranes

Standard or Renografin-purified rickettsiae (10 to 20 mg total protein) were washed, resuspended in buffer A [50 mM potassium phosphate, 10 mM HEPES, pH 7.2, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ATP, and 10 μg/ml RNase and DNase], and disrupted by two passes at 20,000 lb/in² through a french pressure cell (SLM Instruments, Urbana, IL). Residual intact cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C in an SA-600 rotor. The resulting supernatant was removed and the membranes were sedimented by ultracentrifugation at 80,000 rpm for 40 min at 4°C in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA). The membrane pellet was resuspended in buffer A without nucleases and the ultracentrifugation step was repeated. The washed membranes were resuspended in 50 mM potassium phosphate, 10 mM HEPES, pH 7.2, 1 mM DTT, 0.5 mM PMSF, and 10% glycerol at a protein concentration of 10 to 20 mg/ml. The washed membranes were divided into 100-μl aliquots and frozen in liquid nitrogen.

### Solubilization and Reconstitution

Rickettsial ATP/ADP translocase activity was reconstituted into *E. coli* phospholipid liposomes by the octylglucoside dilution procedure of Racker et al. [9]. Thawed rickettsial or uninoculated yolk sac membranes (100 μl of 10 to 20 mg protein/ml) were solubilized on ice with 1.2% octylglucoside in the presence of 0.47% acetone/ether-washed *E. coli* phospholipid, 40% glycerol (vol/vol), 1 mM ATP, 1 mM DTT, 50 mM potassium phosphate, 10 mM HEPES, pH 7.2, in a total extraction volume of 212 μl. Insoluble material was removed by ultracentrifugation as above. The clarified extract was combined with 50 μl of 50 mg/ml bath-sonicated *E. coli* phospholipid liposomes [8] and 4.1 μl 15% octylglucoside. Reconstituted proteoliposomes were formed by a 30-fold dilution into buffer T (50 mM potassium phosphate, 10 mM HEPES, pH 7.2) plus 1 mM DTT, and 0 to 1 mM ATP. Proteoliposomes were collected by centrifugation for 90 min at 35,000 rpm in a Beckman type 35 rotor (4°C), resuspended in 200 μl of the same dilution buffer and frozen in liquid nitrogen.

### Transport Assays

Thawed proteoliposomes (200 μl) were transferred to a 1.5 ml conical microfuge tube. Bath sonication of the proteoliposomes was carried out in 8 to 10 sec intervals (24 to 30 sec, total time) with intermittent Vortex mixing. The bath sonicator (Laboratory Supplies Co., Hicksville, NY) contained 0.02% Triton X-100 and was adjusted for maximum agitation. Extravesicular ATP was removed by AG 1-X8 anion exchange chromatography (1 ml pipette tip plugged with glass wool equilibrated with buffer T). Proteoliposomes flowed through the column, and were collected in a 1-ml volume. Reconstituted proteoliposomes were assayed for ATP/ADP translocase activity by membrane filtration after diluting 250 μl of these proteoliposomes into 650 μl buffer T. Carboxyatractyloside (100 μM) and bongkrekic acid (5 μM) were added when indicated. Transport was initiated by the addition of 100 μl of $\alpha$-$^{32}$P-ATP (10 to 20 μCi) in buffer T. The final ATP concentration ranged between 1 and 10 μM as indicated. The ATP-ATP homologous exchange system was used to prevent charge effects that might have occurred in heterologous exchange and the isotope was commercially available. Transport was carried out at room temperature. The suspension was agitated on a Vortex mixer and at various times 100-μl portions were removed and filtered onto prewetted membrane filters (type GSTF 02500, Millipore Corp., Bedford, MA). The filter was washed once with 5 ml of buffer T. For efflux experiments, proteoliposomes that had equilibrated with $\alpha$-$^{32}$P-ATP were passed back over a AG 1-X8 anion exchange column to remove all extravesicular ATP. These proteoliposomes were then diluted into buffer T with or without 1 mM ATP. Efflux was measured by membrane filtration as described above.

### Miscellaneous Methods

Protein was determined by a modification of the method of Schaffner and Weissmann [10].

### *Chemicals*

$\alpha$-$^{32}$P-ATP (25 Ci/mmol) was obtained from ICN, Biomedicals, Irvine, CA. AG 1-X8 anion exchange resin was purchased from

Bio-Rad Laboratories, Richmond, CA. Acetone/ether-washed *E. coli* phospholipid was purchased from Avanti Polar-Lipids, Birmingham, AL. Bongkrekic acid was a gift from Professor W. Berends (Delft). With the exception of octylglucoside (CalBiochem. La Jolla, CA), all other materials were purchased from Sigma Chemical Co., St. Louis, MO.

## Results

### Renografin-Purified *R. prowazekii* Proteoliposomes

Proteoliposomes formed from membranes of Renografin-purified *R. prowazekii* transported ATP when they had been preloaded with ATP (Fig. 1). Proteoliposomes not preloaded with ATP exhibited no ATP transport activity. The absolute dependence of ATP transport upon exchangeable intravesicular nucleotide indicates that the transport of ATP in proteoliposomes (as in intact rickettsiae) occurred by an obligate exchange mechanism.

Control (no protein) liposomes (prepared by an identical reconstitution procedure with the exception that no rickettsial membranes were included during the extraction procedure) did not transport ATP regardless of the ATP-loading concentration (*data not shown*). These data confirm that the ATP transport activity observed in the reconstituted proteoliposomes was dependent upon rickettsial protein and was not due to nonspecific binding of ATP to the phospholipid vesicles.

Inhibitors of yolk sac mitochondrial ADP/ATP translocase [16], carboxyatractyloside and bongkrekic acid, were tested for the ability to inhibit ATP transport activity in the reconstituted proteoliposomes. The addition of carboxyatractyloside and bongkrekic acid had little or no effect on the reconstituted translocase activity (Fig. 1). These results confirm that the ATP transport activity observed in proteoliposomes reconstituted from Renografin-purified *R. prowazekii* membranes was not due to contaminating mitochondria or mitochondrial membrane fragments.

### Non-Renografin-Purified *R. prowazekii* Proteoliposomes

The ATP transport activity of proteoliposomes reconstituted from standard (non-Renografin-purified) *R. prowazekii* was also investigated (Fig. 1). Proteoliposomes loaded with exchangeable nucleotide mediated the exchange transport of ATP. Interestingly, a significant proportion of this transport activity was sensitive to carboxyatractyloside and bongkrekic acid. The inhibitor-resistant translocase activity remaining in the presence of carboxyatractyloside and bongkrekic acid presumably represented reconstituted rickettsial ATP/ADP translocase activity. The inhibitor-sensitive translocase activity is most likely attributable to a reconstituted eucaryotic translocase from yolk sac mitochondria or mitochondrial membrane fragments in the non-Renografin-purified rickettsial preparation.

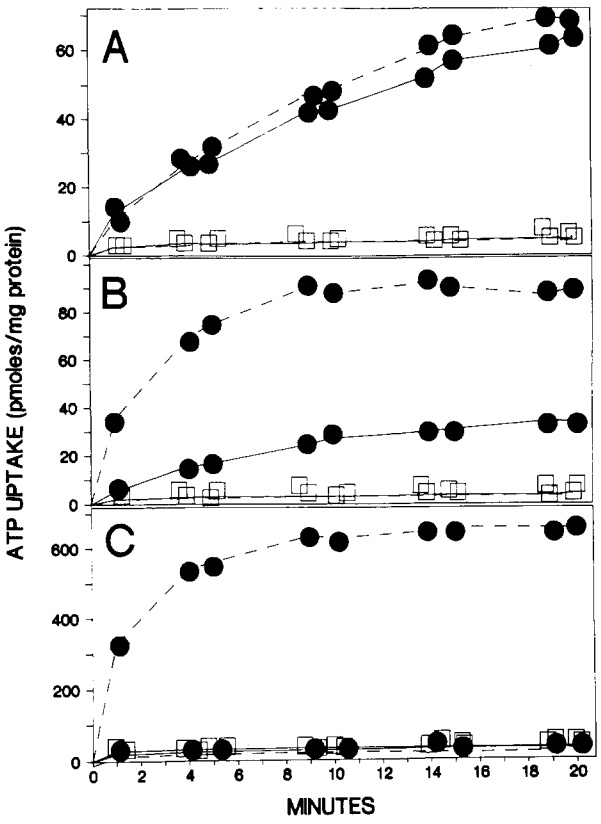


**Fig. 1.** ATP uptake by Renografin-purified *R. prowazekii* proteoliposomes (*A*), standard (non-Renografin-purified) *R. prowazekii* proteoliposomes (*B*), and uninoculated egg yolk sac proteoliposomes (*C*). The transport of ATP (1 $\mu$M) by 1 mM ATP loaded proteoliposomes (●) and by 0 mM ATP loaded proteoliposomes (□) is shown in the presence (solid lines) and the absence (dashed lines) of carboxyatractyloside (100 $\mu$M) and bongkrekic acid (5 $\mu$M)

In order to confirm that the inhibitor-sensitive translocase activity was directly associated with egg yolk sac contaminants, proteoliposomes were prepared using uninoculated egg yolk sac membrane preparations. These proteoliposomes also translocated ATP in exchange for intravesicular ATP (Fig. 1). This translocase activity was inhibited by the addition of carboxyatractyloside and bongkrekic acid. No inhibitor-resistant translocase activity was observed in these proteoliposome preparations. These results indicate that the conditions utilized to reconstitute the rickettsial ATP/

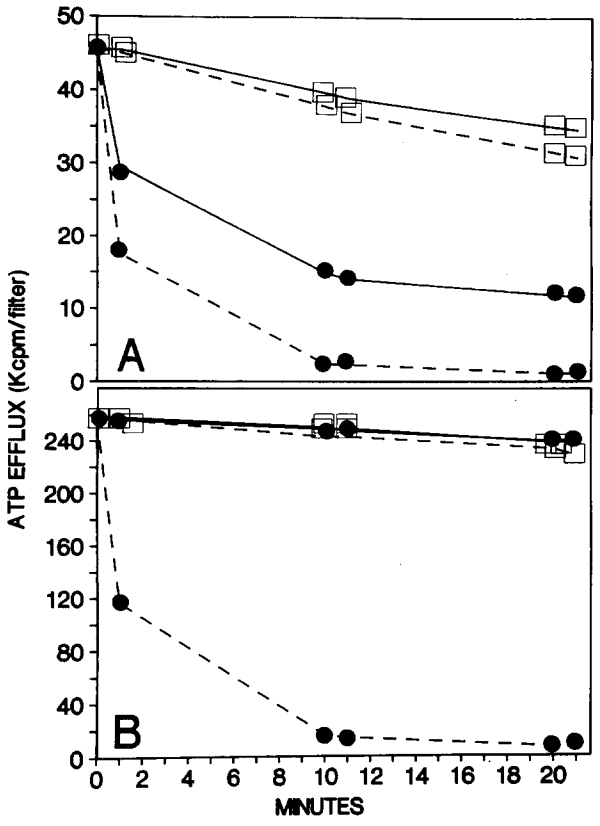


**Fig. 2.** Efflux of ATP from loaded (20 min, 10 $\mu$M) standard (non-Renografin-purified) *R. prowazekii* proteoliposomes (*A*) and from loaded uninoculated egg yolk sac proteoliposomes (*B*). Efflux was assayed in the presence (●) and absence (□) of 1 mM ATP. Efflux in the presence of carboxyatractyloside (100 $\mu$M) and bongkrekic acid (5 $\mu$M) (solid lines) and in their absence (dashed lines) is presented

ADP translocase are also adequate for the reconstitution of a eucaryotic mitochondrial ADP/ATP translocase.

## Efflux Experiments

The efflux of ATP from proteoliposomes prepared using membranes of non-Renografin-purified rickettsiae or from proteoliposomes prepared using uninoculated egg yolk sacs was investigated (Fig. 2). Proteoliposomes were loaded by incubation with $\alpha$-$^{32}$P-ATP, extravesicular ATP was removed by AG 1-X8 anion-exchange chromatography, and efflux experiments were initiated by diluting loaded proteoliposomes into buffer T with or without 1 mM ATP. The efflux of radiolabeled ATP was monitored in both the presence and absence of carboxyatractyloside and bongkrekic acid. Both proteoliposome preparations exhibited no carrier-specific efflux component when diluted into buffer containing no exchangeable nucleotide. A small leak rate from the proteoliposomes could be detected under these conditions. The addition of carboxyatractyloside and bongkrekic acid did not alter these results. On the other hand, dilution of either $\alpha$-$^{32}$P-ATP loaded proteoliposome preparation into buffer T containing 1 mM ATP resulted in almost complete efflux of the intravesicular radiolabeled ATP. Efflux of radiolabeled ATP from proteoliposomes prepared from uninoculated yolk sacs could be blocked by the addition of carboxyatractyloside and bongkrekic acid. This was expected since only the mitrochondrial translocase was present in these proteoliposomes. The proteoliposomes prepared from non-Renografin-purified rickettsiae demonstrated activity typical of both rickettsial and mitochondrial translocases. The addition of carboxyatractyloside and bongkrekic acid to these proteoliposomes partially inhibited the efflux stimulated by 1 mM ATP. However, under these conditions these proteoliposomes exhibited a large efflux component that was inhibitor insensitive and attributable to rickettsial translocase activity. A small but significant proportion of the intravesicular $\alpha$-$^{32}$P-ATP could not be effluxed from these proteoliposomes by 1 mM ATP in the presence of the inhibitors. Presumably, this label was in proteoliposomes that possessed no active rickettsial translocase.

## Specificity of Nucleotide Uptake

Proteoliposomes prepared from membranes of Renografin-purified rickettsiae or proteoliposomes prepared from uninoculated yolk sacs were used to evaluate the substrate specificity of the reconstituted carriers (Table). The ability of various potential substrates to competitively inhibit the translocation of ATP was investigated. For the rickettsial translocase only ADP and ATP were potent competitive inhibitors of ATP translocation. However, ADP, ATP, dADP, dATP and AMP were all able to effectively compete with ATP for the mitochondrial translocase. These results are similar to that reported for intact mitochondria [7] and rickettsiae [16].

## Differential Loading Experiment

The rickettsial ATP/ADP translocase is a specific obligate exchange transport system dependent upon the presence of intravesicular exchangeable nucleotide. Furthermore, since no net accumulation of nucleotide can be achieved by this translocase (based on the properties of the system in intact rickettsiae [16]), the steady-state levels of intravesicular $\alpha$-$^{32}$P-ATP obtained should be dependent upon the initial

**Table** Specificity of nucleotide uptake by the reconstituted rickettsial and mitochondrial translocases

| Inhibitor[a] | Rickettsial[b] translocase (% of control) | Mitochondrial[c] translocase (% of control) |
|---|---|---|
| None | 100 | 100 |
| Adenosine | 112 ± 9.1[d] | 106 ± 0.6[d] |
| AMP | 104 ± 19.9 | 33 ± 0.2 |
| ADP | 31 ± 2.6 | 3 ± 0.1 |
| ATP | 10 ± 0.7 | 2 ± 0.1 |
| dAMP | 123 ± 1.3 | 95 ± 0.1 |
| dADP | 80 ± 12.0 | 4 ± 0.1 |
| dATP | 54 ± 4.0 | 3 ± 0.1 |
| GTP | 79 ± 1.9 | 95 ± 0.2 |
| CTP | 83 ± 9.1 | 95 ± 0.1 |
| UTP | 70 ± 2.7 | 65 ± 0.1 |

[a] The ability of an unlabeled putative inhibitor (1 mM) to inhibit the uptake of $\alpha$-$^{32}$P-ATP (5 $\mu$M) is shown.
[b] The specificity of the *R. prowazekii* ATP/ADP translocase was determined using Renografin-purified *R. prowazekii* proteoliposomes in the presence of carboxyatractyloside (100 $\mu$M) and bongkrekic acid (5 $\mu$M).
[c] The specificity of the mitochondrial translocase was determined using uninoculated egg yolk sac proteoliposomes.
[d] Mean ± standard error of the mean (2 experiments, 2 determinations per experiment).

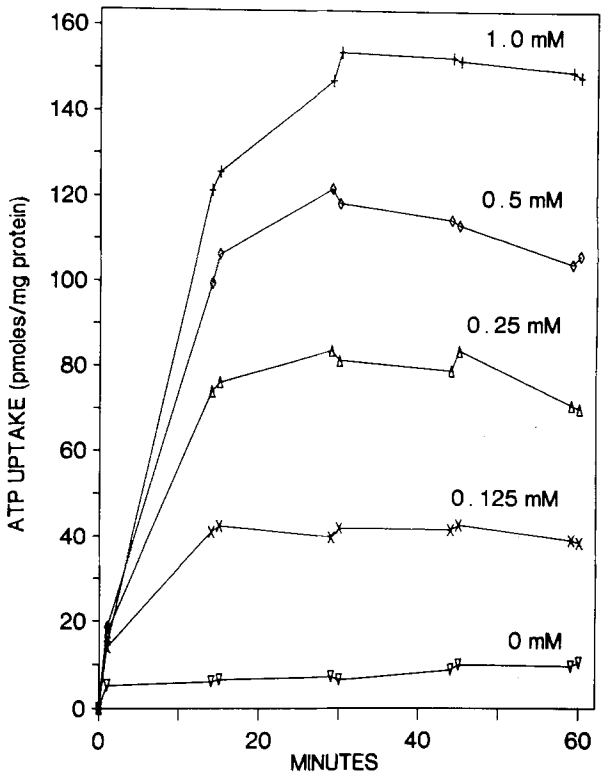


**Fig. 3.** Effect of various intravesicular ATP-loading concentrations on the uptake of ATP (1 $\mu$M) by standard (non-Renografin-purified) *R. prowazekii* proteoliposomes in the presence of carboxyatractyloside (100 $\mu$M) and bongkrekic acid (5 $\mu$M)

loading concentration of intravesicular nonradioactive ATP. The obligate exchange nature of the reconstituted rickettsial translocase was confirmed by determining steady-state $\alpha$-$^{32}$P-ATP levels in proteoliposomes that contained varying levels of intravesicular ATP (Fig. 3). These experiments were conducted in the presence of carboxyatractyloside and bongkrekic acid so that only the rickettsial translocase was active. The time course for uptake of ATP was extended to 60 min to ensure that steady-state levels of $\alpha$-$^{32}$P-ATP were obtained. Interestingly, for unknown reasons, when intravesicular ATP levels greater than 1 mM were utilized an increased proteoliposome leak rate was observed (*data not shown*). For this reason, intravesicular ATP levels were varied between 0 and 1 mM ATP. Extravesicular $\alpha$-$^{32}$P-ATP was added to 1 $\mu$M. Elevations of internal ATP to 1 mM resulted in increases in the final steady-state levels of $\alpha$-$^{32}$P-ATP. The initial rate of ATP transport was similar for all the loaded proteoliposomes. These results can best be explained by an obligate exchange type transport mechanism.

## Discussion

Proteoliposomes prepared from membranes of Renografin-purified *R. prowazekii* transported ATP in exchange for intravesicular ATP. Carboxyatractyloside and bongkrekic acid, specific inhibitors of mitochondrial ADP/ATP translocation, did not inhibit this transport activity. When membranes from non-Renografin-purified rickettsiae were used to reconstitute proteoliposomes both inhibitor-sensitive and inhibitor-resistant translocase activities were identified. Proteoliposomes formed from uninoculated yolk sac membranes demonstrated only the inhibitor-sensitive translocase activity. These data strongly suggest that the inhibitor-sensitive translocase activity represents a reconstituted mitochondrial ADP/ATP translocase activity and that the translocase activity remaining in the presence of carboxyatractyloside and bongkrekic acid represents rickettsial ATP/ADP translocase activity. Interestingly, prior to reconstitution the non-Renografin-purified rickettsial preparation demonstrated very little inhibitor-sensitive translocase activity. This indicates that the rickettsial preparation contained few intact mitochondria. The actual source of the reconstituted mitochondrial translocase is probably mitochondrial membrane fragments present in the non-Renografin-purified rickettsial preparation. During the reconstitution procedure the mitochondrial translocase is solubilized and reconstituted into the phospholipid liposomes.

The purification and reconstitution of the mitochondrial ADP/ATP translocase has been previously described [5, 11]. Triton X-100 was utilized to solubilize the mitochondrial carrier, and reconstitution of the solubilized carrier was achieved utilizing freeze-thaw-sonication procedures. The non-ionic detergent octylglucoside had previously been reported to be destructive to the mitochondrial translocase [5]. In fact, even under the best conditions the Triton X-100 solubilized carrier was still very labile. Utilizing an octylglucoside dilution procedure we have succeeded in reconstituting a carboxyatractyloside and bongkrekic acid-sensitive ADP/ATP translocase from egg yolk sac preparations. The octylglucoside solubilized carrier was very stable, retaining greater than 75% of its activity after 2 hr at 4°C. The possibility does exist that the ADP/ATP translocase activity identified in egg yolk sac preparations is quite different from that of the beefheart mitochondrial ADP/ATP translocase previously characterized. Further studies utilizing an octylglucoside dilution procedure to attempt the reconstitution of the beefheart mitochondrial carrier would be interesting. Perhaps the increased stability of the octylglucoside solubilized carrier would prove useful in certain situations.

The phospholipids utilized in this study were acetone/ether-washed *E. coli* phospholipids. The phospholipid composition of *R. prowazekii* [18] is remarkably similar to that of *E. coli* [14]. In both organisms phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) represent the major phosphatides. The minor phospholipids found in both organisms include phosphatidylserine (PS) and cardiolipin (CL). Significant levels of phosphatidylcholine (PC) have also been identified in rickettsial extracts, but may well represent host cell contamination [18]. Asolectin (14% PC) and egg yolk phospholipids (60% PC) were tested for reconstitution of the rickettsial translocase. The use of these phospholipids yielded very poor recovery of reconstituted translocase activity (*data not shown*). Interestingly, reconstituted mitochondrial translocase activity was also greatest when *E. coli* phospholipid was utilized in the reconstitution procedure. This is understandable since the presence of PE has been shown to be essential for mitochondrial translocase activity [5] and *E. coli* phospholipid contains approximately 60% PE.

The gene coding for the rickettsial ATP/ADP translocase has been cloned and expressed in *E. coli* [6]. DNA sequence analysis of this gene has revealed an open reading frame (ORF) that could encode a hydrophobic protein with a molecular weight of 56,668 and a pI of 10.3 [15]. Ion-exchange chromatography should be useful in purification of this extremely basic protein. Currently, work aimed at increasing the expression of the rickettsial translocase in *E. coli* clones carrying the rickettsial gene is under way. This work is very important, considering the limited amounts of rickettsiae that are available.

In conclusion, conditions for the solubilization and reconstitution of the rickettsial ATP/ADP translocase have been identified. These conditions were also sufficient for the reconstitution of the host cell mitochondrial translocase. The specificity of the reconstituted rickettsial and mitochondrial translocases was investigated and was found to correspond with data from intact rickettsiae and mitochondria. The obligate exchange nature of the reconstituted rickettsial translocase was confirmed. These results indicate that the reconstituted translocases retained the specificity and characteristics associated with their function in natural membranes. The availability of a functional reconstitution procedure provides an assay for the identification and purification of the rickettsial translocase protein and a method for elucidating the molecular function of this transport protein.

This work was supported by Public Health Service grant AI-15035 from the National Institute of Allergy and Infectious Diseases.

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Received 23 March 1989