Identification and Initial Topological Analysis of the *Rickettsia prowazekii* ATP/ADP Translocase

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The *Rickettsia prowazekii* ATP/ADP translocase was identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis using antibodies raised against a synthetic peptide corresponding in sequence to the carboxyl-terminal 17 amino acids of the carrier. Both the translocase of *R. prowazekii* and that expressed by *Escherichia coli* transformants containing the rickettsial gene had an apparent molecular mass of 36,500 Da by SDS-PAGE analysis, a mass considerably less than that deduced from the sequence of the gene. The SDS-solubilized translocase aggregated upon heating at 100°C in the presence of disulfide bond-reducing agents. Similar concentrations of disulfide bond-reducing agents inhibited the exchange transport of adenine nucleotides by both *R. prowazekii* and translocase-expressing *E. coli*. These data suggested that an intramolecular disulfide bond in the translocase was essential for transport activity. The antipeptide antibodies used for identification of the translocase bound preferentially to inside-out membrane vesicles of translocase-expressing *E. coli* relative to right-side-out spheroplasts, thus indicating that the carboxyl terminus of the carrier is located on the cytoplasmic side of the bacterial inner membrane. Protease studies were unable to localize the carboxyl terminus because of the resistance of this region of the native translocase to proteolytic cleavage. These data in conjunction with hydrophobicity analysis were used to construct an initial topological model of the translocase within the cell membrane.

The obligate intracytoplasmic bacterium *Rickettsia prowazekii*, the etiologic agent of the human disease epidemic typhus, grows and multiplies only within the cytoplasm of a eucaryotic cell. This cytoplasm contains metabolites normally unavailable to free-living bacteria, and the rickettsiae have evolved mechanisms to transport many of these large, charged metabolites directly from the host cell's cytoplasmic pools. Such transport systems are very unusual and have never been described in free-living bacteria. *R. prowazekii* has been shown to transport uridine 5'-diphosphoglucose (41), NAD (3), AMP (2), and ADP and ATP (38).

The rickettsial ATP/ADP translocase is an obligate exchange transport system specific for ADP and ATP, so that for every ATP transported into the rickettsiae an ADP molecule must be expelled (38–40). The net result of this exchange is the transport of a high-energy phosphate bond into the rickettsial cell. The gene coding for the *R. prowazekii* ATP/ADP translocase (*tlc* gene) has been cloned and expressed in *Escherichia coli* (23), and the rickettsial translocase has been reconstituted into proteoliposomes (27). The translocation properties of both the cloned gene product and the reconstituted system are the same as those of the translocase in rickettsiae. Analysis of the DNA sequence of the cloned gene revealed a single large open reading frame (ORF) predicted to code for a hydrophobic protein with a molecular mass of 56,668 Da and an isoelectric point of 10.3 (37).

It is interesting that mitochondria possess an analogous system for the exchange of ADP and ATP (21). However, the physiological direction of the exchange is opposite that found in the rickettsiae: the mitochondrial ADP/ATP translocase predominantly catalyzes the exchange transport of cytoplasmic ADP for mitochondrial matrix ATP to serve the

interests of the eucaryotic cell rather than to parasitize it. The mitochondrial transport system is sensitive to specific inhibitors, such as carboxyatractylide (36) and bongkreikic acid (17, 18, 22), none of which have shown an effect on the rickettsial ATP/ADP transport system (27, 38). In addition, comparisons of the highly conserved nucleotide and amino acid sequences of several mitochondrial translocases with those of the rickettsial translocase revealed no homologies (37).

Attempts to identify the product of the *tlc* gene have been largely unsuccessful, presumably because of the poor expression of the *tlc* gene in *E. coli* (28). The identification and characterization of the ATP/ADP translocase will be an important step toward understanding the mechanism of ATP/ADP translocation in *R. prowazekii*. In this study, polyclonal antibodies against a synthetic peptide, corresponding in sequence to the predicted carboxyl-terminal 17 amino acids of the rickettsial ATP/ADP translocase, allowed the identification and characterization of the protein product of the *tlc* gene in the membrane fraction of translocase-expressing *E. coli* and of *R. prowazekii*. These data, in conjunction with DNA sequence data, were used to construct an initial topological model of the ATP/ADP translocase in the bacterial inner membrane.

MATERIALS AND METHODS

Preparation of bacteria and plasmids. *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 (38).

E. coli strains were grown at 37°C in L broth (10) or on LB agar. For antibiotic selection, ampicillin was added to a final

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concentration of 50 µg/ml. Plasmid pMW584 was constructed by inserting a 1.5-kb *EcoRI-HindIII* rickettsial fragment containing the *tlc* gene ORF into plasmid pTZ18R (Pharmacia-LKB, Piscataway, N.J.) directly downstream of the vector *lac* promoter. A portable translation initiation oligonucleotide (Pharmacia-LKB), containing an *E. coli* ribosome-binding site and translation initiation codon, was inserted into the *EcoRI* site directly upstream of the *tlc* gene ORF. The resulting construct was transformed into *E. coli* MOB483 (BL21) (34), generating the recombinant *E. coli* strain MOB603. MOB603 encodes an active ATP/ADP translocase with an expected additional four amino-terminal amino acids. MOB603 and MOB483 were used in ATP uptake determinations. Plasmid pMW384 (37) consists of pUC18 with an inserted 1.6-kb *XbaI-HindII* rickettsial fragment containing the *tlc* gene. *E. coli* DH1 (16) was used as a host for pMW384 and the parent plasmid pUC18, designated strains MOB384 and MOB385, respectively. MOB384 and MOB385 were used to prepare spheroplasts and inside-out (ISO) membrane vesicles for protease susceptibility studies and anti-peptide antibody binding experiments.

Preparation of membrane and soluble fractions from *E. coli* and *R. prowazekii*. *E. coli* or *R. prowazekii* washed cells were suspended to approximately 10 mg of protein per ml in 50 mM potassium phosphate, pH 7.2 (buffer P), containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed by two passages through a French press at 20,000 lb/in². Unlysed whole cells and large debris were removed by centrifugation at 12,200 × *g* for 10 min at 4°C. The cleared lysate was ultracentrifuged at 263,800 × *g* for 30 min in a Beckman TL-100 ultracentrifuge. The resultant supernatant fraction was aliquoted and frozen in liquid nitrogen. The membrane pellet was resuspended in buffer P containing 1 mM PMSF, and the ultracentrifugation step was repeated. The resultant supernatant was discarded, and the membrane pellet was resuspended to approximately 10 mg of protein per ml in buffer P containing 1 mM PMSF and 10% glycerol. Aliquots of the washed membranes were frozen in liquid nitrogen.

Whole cell transport assays. *E. coli* cells grown in L broth containing 50 µg of ampicillin per ml were harvested by centrifugation at 14,000 × *g* for 10 min at 4°C, washed twice with buffer P, and resuspended to approximately 500 Klett units in buffer P containing 0, 1, 5, or 10% β-mercaptoethanol (BME) or dithiothreitol (DTT). Rickettsiae were resuspended to approximately 40 µg of protein per ml in SPG (218 mM sucrose, 3.76 mM KH₂PO₄, 7.1 mM K₂HPO₄, 5 mM glutamic acid, pH 7.0) containing 0, 1, 5, or 10% BME or DTT. After 10 min of preincubation at room temperature, the transport assays were initiated by the addition of 10 µl of radiolabeled nucleotide ([α-³²P]ATP [25 Ci/mmol]) to 390 µl of cell suspension (10 µM ATP, final concentration). At appropriate times, 100-µl aliquots were filtered onto prewetted membrane filters (HAWP 02500; Millipore Corp., Bedford, Mass.) and washed with 10 ml of buffer P (*E. coli*) or SPG (*R. prowazekii*). The filters were dried overnight, and radioactivity determined by liquid scintillation spectrometry.

Preparation of antibodies. A synthetic peptide (NH₂-VKGLNKEYQVLVNKNEK-COOH) that corresponded to the predicted carboxyl-terminal 17 amino acids of the ATP/ADP translocase and contains no linear heptameric epitopes found elsewhere in the translocase sequence was synthesized on an Applied Biosystems (Foster City, Calif.) model 438 peptide synthesizer according to the manufacturer's protocol. This peptide (4 mg in 0.1 M sodium phosphate, pH 7.0) was coupled to the protein carrier thyroglobulin (14 mg)

by addition of glutaraldehyde to 0.25% (vol/vol) for 8 h at room temperature. Excess glutaraldehyde was removed by Sephadex G-75 chromatography.

A pair of female New Zealand White rabbits were injected subcutaneously, along the back, with 540 µg of the peptide-thyroglobulin conjugate emulsified in complete Freund's adjuvant. At 4 and 8 weeks, the rabbits were boosted by subcutaneous injection with peptide-thyroglobulin conjugate emulsified in Freund's incomplete adjuvant. Rabbits were bled at approximately 2-week intervals to obtain serum, which was checked for anti-peptide antibodies by an enzyme-linked immunosorbent assay.

In these assays, peptide (10 µg per well) was bound to the bottom of each well with 0.2% glutaraldehyde in phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 3.4 mM KCl, 170 mM NaCl, pH 7.2) and blocked with bovine serum albumin (BSA; 1 mg/ml in PBS). Dilutions of rabbit serum were added, incubated at 37°C for 2 h, and washed. The second antibody was horseradish peroxidase-conjugated anti-rabbit immunoglobulin G, and the substrate was 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (Sigma Chemical Co., St. Louis, Mo.).

For affinity purification of antibodies, peptide (30 mg) was coupled to 1 ml of epoxy-activated Sepharose 6B (Pharmacia-LKB) in 4 ml of PBS, brought to pH 12.5 with NaOH, and incubated at 37°C for 20 h with gentle agitation. Unreacted coupling sites were blocked by incubation with 1 M ethanolamine for 1 h at 37°C and then washed with PBS, pH 12.5, followed by PBS, pH 7.0. Antiserum (8 to 10 ml) containing anti-peptide antibody activity was applied to the column, followed by a wash with PBS, pH 7.0. Anti-peptide antibodies were eluted from the column with 0.2 M glycine-HCl, pH 2.3, dialyzed against PBS, pH 7.0, and stored at 4°C.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electrotransfer, and immunostaining of blotted proteins. Proteins were solubilized in 2.5% SDS, 0 to 10% BME or DTT, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0. Electrophoresis was performed with 10 to 15% gradient polyacrylamide gels on the Phastsystem electrophoresis system (Pharmacia-LKB). The gels were equilibrated in transfer buffer (3 mM Na₂CO₃, 10 mM NaHCO₃, pH 10.2, 20% methanol, 0.1% SDS) (11, 35) and electrotransferred to Immobilon transfer membranes (Millipore) in a Trans-Blot unit (Bio-Rad Laboratories, Richmond, Calif.) at 125-mA constant current for 40 min at room temperature. The membranes were blocked with 4% BSA in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 30 min at 37°C, washed three times with 0.1% BSA in TBS, and incubated with the anti-peptide antibody preparation diluted 1:1,000 in antibody incubation solution (TBS, 1% BSA, 0.05% Tween 20) for 2 h at 37°C with gentle agitation. To reduce the intensity of nonspecific background staining, the anti-peptide antibody preparation was preadsorbed with an MOB483 extract (final protein concentration, approximately 1 mg/ml) for 1 h at 4°C. After incubation with the first antibody, the membranes were washed and incubated with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma) diluted 1:1,000 in antibody incubation solution for 2 h at 37°C. The membranes were washed three times, and specifically bound antibodies were visualized with the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

Immunoprecipitation of the ATP/ADP translocase. A pellet of isolated membranes (approximately 2 mg of total protein)

from *R. prowazekii* was solubilized with 100 μ l of 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 1% SDS. Insoluble material was removed by centrifugation at $16,000 \times g$ for 10 min at room temperature. The solubilized proteins were diluted into 500 μ l of buffer TD (10 mM Tris-HCl, pH 8.0, 0.85% NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% Triton X-100) and 50 μ l of the anti-peptide antibody preparation or 50 μ l of anti-*R. prowazekii* antiserum, mixed, and incubated at 4°C overnight. A suspension of protein A-Sepharose 4B-CL (34 mg; Pharmacia-LKB) was added to the antigen-antibody solution and incubated with mixing for 1 h at 4°C. The complex was collected by centrifugation at $16,000 \times g$ for 10 min at room temperature, washed three times with buffer TD, and resuspended in 2.5% SDS–5.0% BME–1 mM EDTA–10 mM Tris-HCl, pH 8.0. After centrifugation, the supernatant fluid was analyzed by SDS-PAGE and immunoblotting.

Binding of antibody to and proteolytic digestion of spheroplasts and ISO membrane vesicles. Spheroplasts were prepared from strains MOB384 and MOB385 grown to mid-log phase in L broth plus ampicillin (20). ISO membrane vesicles were prepared by controlled lysis of spheroplasts by a single pass at 7,000 lb/in² through a French press (13, 14). Residual intact cells were removed by centrifugation at $14,000 \times g$ for 10 min at 4°C. Spheroplasts and ISO membrane vesicles were used immediately without freezing to determine the sidedness and protease accessibility of the carboxyl terminus.

Spheroplasts and ISO membrane vesicles were suspended without washing to a final total protein concentration of approximately 1.0 mg/ml in TBS buffer containing 25% sucrose, 1% BSA, and 0.05% Tween 20. Portions (300 μ l) of these preparations were incubated with 100 μ l of a 1:1,000 dilution of the anti-peptide antibody in the same buffer for 1 h at room temperature in polycarbonate ultracentrifuge tubes (Beckman Instruments, Inc., Fullerton, Calif.). The ISO membrane vesicle suspensions were sedimented at $263,800 \times g$ for 20 min, and the spheroplast preparations were sedimented at $2,600 \times g$ for 10 min in the TL-100 ultracentrifuge at room temperature. The ISO membrane vesicles and the spheroplasts were resuspended in 400 μ l of TBS buffer containing 25% sucrose, 1% BSA, 0.05% Tween 20, and ¹²⁵I-protein A (0.36 μ Ci/150 μ l). The suspensions were incubated for 1 h at room temperature and centrifuged as described above, and the radioactivity in the supernatant fluid and pellet was measured in a liquid scintillation counter (Pharmacia-LKB).

Spheroplasts and ISO membrane vesicles (400 μ l of 1.0 mg of protein per ml) were incubated for 1 h at room temperature with 100 μ g of proteinase K (Sigma type XI; 18.5 U/mg of protein) or α -chymotrypsin (Sigma TLCK-treated; 60 U/mg of protein), and the reactions terminated by the addition of PMSF to a final concentration of 2 mM. The treated spheroplasts or ISO membrane vesicles were collected by ultracentrifugation at $263,800 \times g$ for 30 min at 4°C.

Protein determination. Protein was determined by a modification of the method of Schaffner and Weissmann (30) or by the BCA method (Pierce, Rockford, Ill.).

RESULTS

Identification of the ATP/ADP translocase on immunoblots of SDS-polyacrylamide gels and by immunoprecipitation. Affinity-purified anti-peptide antibodies (325 μ g of protein per ml) prepared to the deduced carboxyl-terminal heptadecimer (37) of the rickettsial ATP/ADP translocase were used to

FIG. 1. Identification of the ATP/ADP translocase by SDS-PAGE and immunoblot analysis. Soluble (S) and membrane (M) fractions of *R. prowazekii* (Rp) and *E. coli* MOB384 and MOB385 were solubilized at room temperature in SDS sample buffer (2.5% SDS, 5% BME, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and electrophoresed on 10 to 15% SDS-polyacrylamide gels. Proteins were silver stained (A) or transferred to Immobilon membranes and immunostained (B). The position of the ATP/ADP translocase is indicated by the arrow in panel B. Molecular weight standards (MW STD): lysozyme (14 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa), and phosphorylase (92 kDa).

identify and characterize the ATP/ADP translocase. An immunoreactive protein band, migrating with an apparent molecular mass of 36,500 Da, was found in the membrane fraction of MOB384 (a translocase-expressing *E. coli* strain) and *R. prowazekii* by immunoblot analysis (Fig. 1). No corresponding immunoreactive band was identified in the membrane of the negative control *E. coli* MOB385 or in the soluble fractions of any of the organisms. No band corresponding to the immunostained band was identified in the silver-stained gel.

The ATP/ADP translocase was immunoprecipitated from solubilized membranes of *R. prowazekii* with the anti-peptide antibody preparation, separated by SDS-PAGE, and visualized by silver staining or immunoblot analysis (Fig. 2). A 36,500-Da band was identified both in the silver-stained gel (Fig. 2A) and in the immunoblot analyzed (Fig. 2B). The

FIG. 2. Immunoprecipitation of the ATP/ADP translocase from *R. prowazekii* membranes. Solubilized rickettsial proteins were immunoprecipitated with the anti-peptide antibody (ANTI-PEP Ab) or with a polyclonal anti-*R. prowazekii* antiserum (ANTI-Rp Ab) and electrophoresed on 10 to 15% SDS-polyacrylamide gels. Proteins were silver stained (A) or transferred to Immobilon membranes and immunostained (B). The second and third lanes are duplicates. The position of the ATP/ADP translocase is indicated by the arrow. Molecular weight standards (MW STD) are as in Fig. 1.

FIG. 3. Effect of heating and disulfide bond-reducing agents on aggregation of the ATP/ADP translocase. Prior to SDS-PAGE analysis, rickettsial membrane samples were solubilized with SDS sample buffer (2.5% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) containing 0 or 5% BME. The samples were not heated or were heated at 100°C for 1 or 5 min prior to electrophoresis. Proteins were transferred to Immobilon transfer membranes and immunostained. The position of the ATP/ADP translocase is indicated by the arrow. Times are indicated in minutes.

heavy chain of the precipitating rabbit immunoglobulin, as expected, was also seen on the immunoblot. The ATP/ADP translocase was not immunoprecipitated by the antiserum against whole *R. prowazekii*, indicating the sparsity of anti-ATP/ADP translocase antibodies in this antiserum (Fig. 2). These data indicate that the 36,500-Da immunoreactive band is the rickettsial ATP/ADP translocase and that it is found in the membrane fraction of the translocase-expressing *E. coli* strain MOB384 and *R. prowazekii*.

Effect of disulfide bond-reducing agents on aggregation of the ATP/ADP translocase. Electrophoretic analysis of proteins solubilized by heating in a boiling water bath for 1 to 5 min in the presence of SDS and a disulfide bond-reducing agent, such as BME, is a standard procedure for separating proteins on the basis of molecular weight (24). However, attempts to analyze rickettsial membrane samples by this technique resulted in the complete aggregation of the translocase and its failure to enter the separating portion of an SDS-polyacrylamide gel (Fig. 3). Aggregation of the translocase was more pronounced as the time of sample heating increased, with complete aggregation of the translocase occurring at 5 min. Rickettsial membrane samples that were solubilized at room temperature showed no aggregation of the translocase. In addition, the translocase did not aggregate upon heating if the disulfide bond-reducing agent was omitted from the solubilization buffer. A concentration of either 5% DTT or 5% BME was sufficient to promote aggregation of the translocase upon heating; however, 1% DTT or 1% BME was insufficient to promote the aggregation phenomenon. As a control for the specific disulfide bond-reducing functions of BME and DTT, chemically similar but nonreducing analogs (butanediol, *trans*-4,5-dihydroxy-1,2-dithiane, threitol, and ethylene glycol) were added at a 10% concentration. Addition of the less polar analogs, butanediol and *trans*-4,5-dihydroxy-1,2-dithiane, caused aggregation when the samples were heated; on the other hand, addition of the more polar analogs, threitol and ethylene glycol, did not cause aggregation.

Effect of disulfide bond-reducing agents on the transport activity of the ATP/ADP translocase. The addition of high concentrations (5% or 10% BME or DTT) of disulfide bond-reducing agents to translocase-expressing *E. coli* or to *R. prowazekii* completely inhibited the translocation of ATP, while lower concentrations (1% BME or 1% DTT) had little effect on ATP transport (Fig. 4). The inhibition was readily

FIG. 4. Effects of BME and DTT on the uptake of ATP by *R. prowazekii* and translocase-expressing *E. coli*. The uptake of ATP at 10 min by *R. prowazekii* (■) and *E. coli* MOB603 (■) and MOB483 (▨) in the presence of the indicated concentration of disulfide bond-reducing agent was determined. Control values were determined in the absence of disulfide bond-reducing agents. The effect of removal of disulfide bond-reducing agents from BME- or DTT-treated MOB603 upon ATP transport is presented as follows: CTL-BME, initially treated without disulfide bond-reducing agents and then treated with 5% BME; BME-BME, 5% BME treatment followed by 5% BME treatment; BME-CTL, 5% BME treatment followed by treatment without disulfide bond-reducing agents; CTL-DTT, initially treated without disulfide bond-reducing agents and then treated with 5% DTT; DTT-DTT, 5% DTT treatment followed by 5% DTT treatment; DTT-CTL, 5% DTT treatment followed by treatment without disulfide bond-reducing agents. The error bars indicate the standard deviation for duplicate time points.

reversed by sedimentation of the treated cells and resuspension of these cells in medium without the agents (Fig. 4). Since the activity of the ATP/ADP translocase is not inhibited by metabolic poisons, dissipation of the membrane potential, or *N*-ethylmaleimide (NEM) (38), secondary effects of BME or DTT on the translocase are unlikely. Furthermore, none of the chemically similar but nonreducing analogs (butanediol, threitol, and ethylene glycol) appreciably inhibited transport (*trans*-4,5-dihydroxy-1,2-dithiane is insoluble in aqueous buffers and could not be tested). These data suggest that maintenance of a disulfide bond in the translocase is necessary for the proper functioning of the transport system and that the removal of the reducing agent leads to the restoration of this critical disulfide bond and transport activity.

Determination of the protease accessibility of the carboxyl terminus of the translocase in spheroplasts and ISO vesicles of MOB384. Spheroplasts which maintain a right-side-out (RSO) orientation (42) and high-pressure lysis membrane vesicles which are largely oriented ISO (13, 14) were prepared from the translocase-expressing *E. coli* strain MOB384, treated with chymotrypsin or proteinase K, and subjected to SDS-PAGE and immunoblotting with the anti-

FIG. 5. Susceptibility of the ATP/ADP translocase to proteolytic digestion in spheroplasts and ISO membrane vesicles. Spheroplasts and ISO vesicles of the translocase-expressing *E. coli* strain MOB384 were treated with proteinase K (PK), chymotrypsin (CT), or no additions (CONTROL). Immunoblots of protease-treated and control spheroplasts or ISO vesicles and of an MOB384 membrane preparation (MOB384) are presented. The position of the full-size ATP/ADP translocase is indicated by the arrow and is present in the control samples and in the MOB384 membrane sample. Low-molecular-weight immunoreactive digestion products were present in the CT- and PK-treated spheroplast and ISO vesicle preparations. Molecular weight standards (MW STD) are as in Fig. 1.

peptide antibody preparation (Fig. 5). In both vesicle preparations, protease treatment hydrolyzed the translocase so that the 36,500-Da band was absent. Furthermore, low-molecular-weight carboxyl-terminal peptide fragments were generated that were still reactive with the antibodies but were too small to allow accurate molecular weight determinations in the gel system used. Although the carboxyl-terminal epitopes recognized by the anti-peptide antibodies were not susceptible to proteolytic degradation in spheroplasts or ISO membrane vesicles, complete degradation of the immunoreactive portion of the translocase by chymotrypsin or proteinase K was readily demonstrated in the membrane preparations solubilized by 1% Triton X-100 (data not shown). The resistance of the carboxyl-terminal region to proteolytic degradation in native membranes in either orientation prevented us from obtaining topological data by this technique.

Antipeptide antibody binding to spheroplasts and ISO vesicles of MOB384 and MOB385. The binding of anti-peptide antibodies to spheroplasts and ISO membrane vesicles of translocase-expressing *E. coli* was measured to determine the accessibility and hence location of the carboxyl terminus. ISO membrane vesicles prepared from the translocase-expressing *E. coli* strain MOB384 that were probed with the anti-peptide antibody and 125 I-protein A bound at least eight times more radioactivity than did the RSO spheroplasts (Fig. 6). Neither spheroplasts nor ISO vesicles prepared from the negative control *E. coli* MOB385 bound significant amounts of radioactivity. These data indicate that the carboxyl terminus of the translocase is exposed on the cytoplasmic face of the inner membrane.

The accessibility of the carboxyl-terminal region to anti-peptide antibodies in ISO membrane vesicles was intriguing since this region appeared to be inaccessible to proteases in identical preparations. Protease-treated ISO membrane vesicles were probed with anti-peptide antibodies and 125 I-protein A to determine whether the epitopes recognized by the anti-peptide antibodies had been hydrolyzed (Fig. 6). The anti-peptide antibodies bound to both protease-treated and

FIG. 6. Binding of anti-peptide antibodies to spheroplasts and ISO membrane vesicles. (A) Binding of 125 I-protein A to spheroplasts and ISO vesicles of the translocase-expressing *E. coli* strain MOB384 (■) and the translocase-negative strain MOB385 (□) after treatment with the anti-peptide antibodies (+Ab) or in the absence of anti-peptide antibody treatment (-Ab). (B) Binding of anti-peptide antibodies to control, proteinase K (PK)-, or chymotrypsin (CT)-treated ISO vesicles of the translocase-expressing *E. coli* strain MOB384 (■) and the translocase-negative strain MOB385 (□).

control MOB384 ISO membrane vesicles in equal amounts. As expected, the anti-peptide antibody did not bind to protease-treated MOB385 ISO membrane vesicles. These data indicate that the anti-peptide antibody has accessibility to a protease-resistant region of the carboxyl terminus of the ATP/ADP translocase.

DISCUSSION

Antibodies to a 17-amino-acid synthetic peptide, representing the deduced carboxyl-terminal sequence of the rickettsial ATP/ADP translocase, were used to identify the translocase in immunoblots of translocase-expressing *E. coli* strains and *R. prowazekii*. This immunostained band was found only in the membrane fraction of *E. coli* transformants containing the rickettsial *tlc* gene and in *R. prowazekii* itself and had an apparent molecular mass of 36,500 Da by SDS-PAGE analysis. Similarly, when the translocase was immunoprecipitated from SDS-solubilized *R. prowazekii* membranes with the anti-peptide antibody preparation and protein A-Sepharose, a single polypeptide band with the same apparent molecular mass was identified in both silver-stained and immunostained preparations.

Analysis of the translocase by standard SDS-PAGE and immunoblotting techniques was complicated by the atypical behavior of this protein. The translocase aggregated upon heating at 100°C for 3 to 5 min in standard SDS sample buffer and failed to enter the separating portion of an SDS-polyacrylamide gel. Irreversible aggregation of other hydrophobic membrane proteins upon heating at 100°C has been reported. For example, in *E. coli*, the lactose permease (6), the proline carrier (15), the SecY protein (19), and the RodA protein (33) all aggregate upon heating at 100°C. An unusual facet of the aggregation of the rickettsial translocase is the

requirement for high concentrations of disulfide bond-reducing agent, butanediol or *trans*-4,5-dihydroxy-1,2-dithiane. The translocase aggregated upon heating in the presence of 5 to 10% BME or DTT but did not aggregate when the concentration was reduced to 1% or less. A related phenomenon has been reported for the β -adrenergic receptors of eucaryotic cells, in which the presence of intramolecular disulfide bridges in the receptor was established by demonstrating that chemical reduction of these bonds decreased the electrophoretic mobility of these proteins (25, 26). On the other hand, the temperature-dependent formation of high-molecular-weight complexes in the presence of BME has also been reported for the cystineless reaction center proteins of *Rhodospseudomonas sphaeroides* (32). Thus, these data do not unambiguously indicate that the reduction of a disulfide bond in the ATP/ADP translocase results in a conformational change that predisposes the translocase to denaturation and aggregation upon heating.

However, the data strongly suggest that a disulfide bond within the membrane is necessary for maintaining the carrier in an active conformation. The translocation of ATP was inhibited by high concentrations of reducing agents but was not inhibited by the nonreducing analogs of these agents. Interestingly, analysis of the predicted amino acid composition of the ATP/ADP translocase revealed the presence of only two cysteines in the coding region for this protein (Cys-37 and Cys-85). Reduction of this single intramolecular disulfide bridge may result in an unfolding of the detergent-solubilized protein which exposes hydrophobic domains that can interact to form a large, insoluble aggregate. In addition to predisposing the solubilized translocase to heat denaturation and aggregation, the destabilization of the native translocase may interfere with substrate binding or with the translocation process itself.

The inhibition of adenine nucleotide translocation by disulfide bond-reducing agents was readily reversed by the removal of the reducing agent from the inhibited cells. The sulfhydryl alkylating reagent NEM was unable to prevent the reversal of inhibition upon removal of the reducing agent, presumably because of its inability to reach the susceptible sulfhydryl groups prior to reoxidation and reformation of a disulfide bond. NEM had no effect on ATP transport, which indicates that accessible, free sulfhydryls were not essential for translocase activity. The high concentrations of disulfide bond-reducing agent required to inhibit the translocation process indicate that the disulfide bond of the translocase is relatively resistant to reduction when native and within the lipid bilayer.

The value of 36,500 Da estimated from immunoblots and immunoprecipitations differs considerably from that of 56,668 Da calculated from the DNA sequence data (37). In theory, proteolysis could have occurred, but would be limited to removal of the first 36 amino acids (to retain Cys-37 for the disulfide bond) and the last 10 to 13 amino acids at the carboxyl terminus (to retain the amino acids necessary for an epitope). This truncated protein would still be greater than 50 kDa. The most likely explanation is that the apparent low-molecular-weight value by SDS-PAGE analysis is due to excessive binding of SDS to hydrophobic regions of the protein, a phenomenon that is not uncommon with hydrophobic membrane proteins (6, 8, 9).

The use of impermeant, high-molecular-weight probes, such as antibodies and proteases, to obtain information on the orientation of membrane-spanning polypeptide chains is an important tool for determining the topological arrangement of an integral membrane protein. An attractive ap-

proach for determining the orientation of a membrane protein bilayer is to synthesize peptides on the basis of the deduced sequence and raise antibodies to those peptides which correspond to specific hydrophilic portions of the protein. In the literature, such site-directed antibodies have localized the reactive epitopes at either side of the membrane. A carboxyl-terminal sequence was chosen for the synthesis of a peptide for both the lactose carrier and the melibiose carrier of *E. coli* (5, 31). Site-directed antibodies produced against these peptides bound only to the surface of ISO vesicles and not to RSO vesicles, indicating that the carboxyl terminus of both of these proteins is exposed on the cytoplasmic face of the membrane (5, 31). In contrast, the monoclonal antibody 4b1 bound only to spheroplasts or RSO membrane vesicles, indicating that this reactive epitope of the lactose permease was located on the periplasmic side of the membrane (7).

The carboxyl-terminal region of the ATP/ADP translocase was an obvious candidate for synthesis of an immunogenic peptide since this region has a hydrophilic sequence that may exist as an extension from the hydrophobic core of the translocase (37). These anti-peptide antibodies bound to ISO vesicles of the translocase-expressing *E. coli* strain MOB384 but did not bind to RSO spheroplasts of this same strain or to either spheroplasts or ISO vesicles of the negative control *E. coli* MOB385. These data demonstrate that the carboxyl terminus of the translocase is exposed on the cytoplasmic side of the membrane.

The susceptibility of the ATP/ADP translocase to proteolytic degradation in both spheroplasts and ISO vesicles was determined by treating these preparations with various proteases and analyzing the treated vesicles by SDS-PAGE and immunoblot analysis with the anti-peptide antibodies. As expected, both chymotrypsin and proteinase K partially degraded the translocase in spheroplasts and ISO vesicle preparations, as judged by the disappearance of the 36,500-Da immunoreactive band in immunoblot analysis. Surprisingly, in both preparations the immunoreactive epitopes of the carboxyl terminus were protected. Since at least a portion of the carboxyl terminus must be exposed on the cytoplasmic side of the cell membrane for the anti-peptide antibodies to bind, ISO membrane vesicles were treated with either proteinase K or chymotrypsin to remove any exposed protease-susceptible epitopes from the vesicles and then reacted with anti-peptide antibodies to determine whether the epitopes recognized by the antibodies had been digested. The anti-peptide antibodies bound equally to both protease-treated and control ISO vesicles of MOB384. This indicates that the carboxyl-terminal portion of the ATP/ADP translocase is accessible to the anti-peptide antibodies but not to these proteases.

A topological model for the ATP/ADP translocase, based on hydropathy analysis of the predicted ATP/ADP translocase amino acid sequence (37), predicts 12 hydrophobic α -helical transmembrane regions, with 13 extramembrane hydrophilic extensions connecting the transmembrane regions (Fig. 7). The model is presented with the carboxyl terminus in a cytoplasmic orientation as directed by the anti-peptide antibody binding data. A disulfide bond is indicated between the cysteine residue at position 37 and the cysteine residue at position 85, which are presumed to be located between the first two transmembrane regions. The large number of charged groups in the extramembrane domains provide a hydrophilic region for interaction with the aqueous phase and with substrates. This model of the ATP/ADP translocase is similar to that predicted for a

FIG. 7. Schematic model of the ATP/ADP translocase within the lipid bilayer. The predicted 12 membrane-spanning, alpha-helical, hydrophobic segments of the ATP/ADP translocase are drawn inside the 12 cylinders. The hydrophilic portions of the translocase are located on either side of the lipid bilayer. The single disulfide bond (==) is indicated between the first two membrane-spanning alpha-helical segments. The basic amino acids and the acidic amino acids are in heavy and light squares, respectively.

number of other transport proteins from both procaryotes and eucaryotes. In each case, the model suggests a hydrophobic integral membrane protein, usually with 12 transmembrane segments, and a cytoplasmic extramembrane domain of significant size. These proteins, which include the lactose permease (12), are suggested to function as monomers, as opposed to a related group of carriers which possess six transmembrane segments and appear to function as dimers (1, 29). This second class of carriers includes the mitochondrial ATP/ADP translocase (4) and several other mitochondrial and chloroplast transport systems. Interestingly, a full complement of 12 membrane-spanning segments often appears to be necessary for functional transport activity. The conformation of this general design requires more detailed investigations of transmembrane topology and eventually the construction of definitive models based on X-ray crystallographic studies.

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