Cysteine-Scanning Mutagenesis and Thiol Modification of the *Rickettsia prowazekii* ATP/ADP Translocase: Evidence that TM VIII Faces an Aqueous Channel[†]

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ABSTRACT: The contribution of transmembrane region VIII of the *Rickettsia prowazekii* ATP/ADP translocase to the structure of the water-filled channel through which ATP is transported was evaluated from the accessibility of three hydrophilic, thiol reactive, methanethiosulfonate reagents to a library of 21 single-cysteine substitution mutants expressed in *Escherichia coli.* A negatively charged reagent (MTSES) and two positively charged reagents (MTSET and MTSEA) were used. Mutants Q323C and G327C did not tolerate cysteine substitution and were almost completely deficient in ATP transport. The remaining mutants exhibited 25−226% of the cysteine-less parent's transport activity. Five patterns of inhibition of ATP transport by the MTS reagents were observed. (i) ATP transport was not inhibited by any of the three MTS reagents in mutants Q321C, F324C, A332C, and L335C and only marginally in F333C. (ii) Transport activity of mutants F322C, Q326C, and A330C was markedly inhibited by all three reagents. (iii) ATP transport was inhibited by MTSEA in only the largest group of mutants (M334C, I336C, G337C, S338C, N339C, I340C, and I341C). (iv) Transport activity was inhibited by MTSET and MTSEA, whereas high concentrations of MTSES were required to inhibit mutants W328C, V329C, and I331C. However, mutant W328C could be inhibited by MTSES in the presence of sub-K_m concentrations of the substrate. (v) ATP transport by mutant Y325C was unaffected by MTSEA, but inhibited ∼50% by MTSET and MTSES. Transport of ATP protected mutants (F322C, W328C, V329C, A330C, and I331C) from MTS inhibition. Mutants in the half of TM VIII that is closest to the cytoplasm were not inhibited well by MTSES or MTSET in either whole cells or inside-out vesicles. The results indicate that TM VIII makes a major contribution to the structure of the aqueous translocation pathway, that the accessibility to impermeant thiol reagents is influenced (blocked or stimulated) by substrate, and that there is great variation in accessibility to MTS reagents along the length of TM VIII.

The ATP/ADP translocase of *Rickettsia prowazekii* facilitates the exchange of host cell ATP and rickettsial ADP and is, hence, a source of energy and not adenylate. *R. prowazekii,* the agent of epidemic typhus, is an obligate intracellular alpha proteobacteria with a Gram-negative architecture that lives within the cytoplasm of a eukaryotic host cell, unbound by any host-derived membrane structure (for reviews, see refs *1* and *2*). The direct cytosolic access afforded to this unusual bacterium resulted in the evolution of transporters of metabolic products that are available in the host cell's cytoplasm. The ability of bacteria that occupy the extracellular niche to transport cytosolic metabolites is unusual because these compounds are typically large, charged, and rarely available. The *R. prowazekii* ATP/ADP translocase gene (*tlc1*) has been characterized both in purified rickettsiae and in *Escherichia coli* that are expressing a plasmid-borne, rickettsial *tlc1* gene (*3*−*11*).

Structural studies of membrane-bound proteins have typically been hindered by the difficulty of obtaining them in a form that is suitable for X-ray crystallography. Furthermore, the amount of translocase expressed heterologously in *E. coli* is usually ∼1 mg of protein per liter of culture, and all attempts to markedly improve expression, including making a synthetic *tlc1* gene with the preferred *E. coli* codon usage, have been unsuccessful (*3*, *4*). Therefore, we have employed genetic and alternative biochemical approaches for studies of translocase topology. We have provided evidence that the ATP/ADP translocase has 12 transmembrane regions and that the seven odd-numbered hydrophilic loops are on the cytoplasmic side of the membrane and the six even-numbered loops are on the periplasmic side (Figure 1) (*3*, *4*). This was based on measurements of both β-galactosidase and alkaline phosphatase activity in dual *pho-lac* reporters fused to the *tlc1* gene. The ATP/ADP translocase is at present the sole member of a distinct transport protein family in the classification scheme of Saier (*12*). ATP/ADP translocases with similar functions and amino acid sequences have now been identified in all obligate intracellular bacteria investigated to date, ranging from the human pathogens, rickettsiae and chlamydiae, to the protistic endosymbionts, holospora and caedibacter (*13*). Curiously, although not present in cyano-

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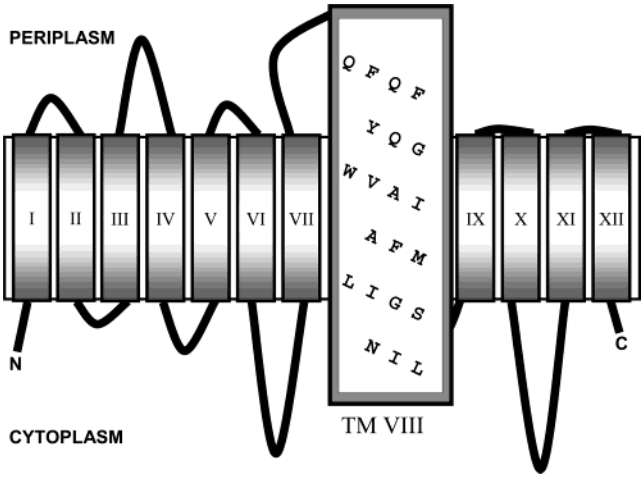

FIGURE 1: Model of the rickettsial ATP/ADP translocase. The hydrophilic loops connecting the 12 transmembrane regions have lengths proportional to the number of residues in each loop. The N- and C-termini and their periplasmic or cytoplasmic orientation are indicated.

bacteria, this translocase is present in the plastids of plants ranging from land plants to red algae (*13*). Most significantly, although rickettsiae are evolutionarily the bacteria closest to the mitochondria, the mitochondrial ADP/ATP exchanger shows no homology in sequence and little similarity in structure with the rickettsial ATP/ADP translocase and has a reversed function; i.e., the parasite takes ATP from the host cell, whereas the mitochondrion provides ATP to the host cell (*14*).

In the study presented here, we determined the access of the amino acid residues in transmembrane region VIII (TM VIII)[1] to an aqueous channel, a channel that we presume to be the translocation pathway across the membrane for ATP and ADP. To this end, a previously constructed, cysteine-less mutant of the *R. prowazekii tlc1* gene without any affinity tags or other modifications (*3*) was subjected to site-directed mutagenesis, resulting in a library of mutants in TM VIII each containing a single-cysteine substitution. These single-cysteine-containing *tlc1* mutants were then exposed to specific, hydrophilic, and highly thiol reactive reagents [sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES), 2-(trimethylammonium)ethyl methanethiosulfonate bromide (MTSET), and 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA)] to determine if these MTS reagents have access to residues in TM VIII. MTS-inhibitable residues would be candidates for lining the water-filled channel through which ATP is translocated. Mutants with accessible residues were then tested to determine whether the presence of the substrate in the translocation pathway would protect these residues from the hydrophilic reagents. Strategies similar to this have been employed in examining the membrane topology of transport proteins in other families of transport proteins (*15*−*25*). All of these studies must assume that the substitution of a cysteine for the native residue does not change the native conformation of the protein.

[1] Abbreviations: TM VIII, transmembrane region VIII; MTS, methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate bromide; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; M9CGAp^{100}, minimal medium supplemented with 0.1% casamino acids, 0.2% glucose, and 100 μg/mL ampicillin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; KP$_i$, potassium phosphate buffer; ISO, inside-out; LBAp^{100}, Luria-Bertani medium supplemented with 100 μg/mL ampicillin.

MATERIALS AND METHODS

Growth Conditions and Reagents Used in This Study. Bacteria were grown in Luria-Bertani broth or M9 minimal medium supplemented with 0.1% casamino acids, 0.2% glucose, and 100 μg/mL ampicillin (M9CGAp^{100}) at 37 °C with aeration. The MTSES, MTSET, and MTSEA compounds were purchased from Toronto Research Chemical Inc. (North York, ON).

Construction of Single-Cysteine Substitution Mutants. To construct a bank of single-cysteine substitution mutants representing every amino acid residue of TM VIII, we utilized a previously constructed pET11a expression vector containing the *R. prowazekii tlc1* gene in which both native cysteine residues had been mutated to alanine (*3*). In addition, a *Pst*I restriction site was removed from this vector, leaving a single site in the *tlc1* gene. TM VIII was selected for this study because of the unique flanking *Pst*I and *Bst*BI sites that allowed us to subclone PCR-mutagenized fragments back into pET11a-*tlc1* which abrogates the need to sequence the entire gene to confirm the presence of the mutation. The PCR mutagenesis method of Ito (*26*) was used to construct single-cysteine mutants Q321C to L335C and cysteine mutants I336C to L341C were constructed using a megaprimer PCR method (*27*). Mutagenized constructs were transformed into a chemically competent DH5α strain [F$^-$ 080d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_K^-m_K^+$) *phoA supE44* 1- *thi-1 gyrA96 relA1*] of *E. coli*. Transformants were selected on LBAp^{100} agar plates, and plasmids were isolated and transformed into a chemically competent BL21(DE3) strain of *E. coli* so that expression from the T7 promoter could be induced by the addition of IPTG.

ATP Uptake Assays. The uptake assay was essentially as described previously (*28*). Overnight cultures of *E. coli* BL21(DE3) harboring pET11a-*tlc1* wild-type (cysteine-less) and mutant plasmids were grown in M9CGAp^{100}, subcultured into fresh medium, and grown to an OD_{600} of 0.4, at which point 1 mM IPTG was added. After induction for 1 h, bacteria were collected by centrifugation, washed once in an equal volume of 50 mM potassium phosphate buffer (KP$_i$, pH 7.5), and resuspended in KP$_i$ to $^1/_{20}$ of the original volume (all operations on ice). These bacteria were diluted 1:10 into room-temperature KP$_i$ followed by the addition of [α-^{32}P]ATP (final concentration of 50 μM, 1−2 μCi/mL). The rate of transport was determined by transferring 100 μL aliquots 1, 2, 9, and 10 min after the addition of ATP to wetted filter membranes under vacuum, washing once with 5 mL of KP$_i$, and determining the intracellular radioactivity by scintillation spectrometry. ATP uptake was normalized on the basis of the total protein present in the sample.

Immunoblot Assays. Our initial uptake assays determined that a cysteine substitution at positions Q323 or G327 resulted in near-complete ablation of transport activity. Further, a cysteine substitution at position F324 resulted in a hyperactive translocase mutant. To determine if differences in protein expression could account for these mutant

phenotypes, we determined expression levels using immunoblot analysis. Bacterial membranes from cultures grown as described above were collected by ultracentrifugation of bacteria lysed with a French pressure cell and suspended in 0.1% SDS. The membrane protein was separated by SDS−PAGE (4 to 20% gel) and transferred to a polyvinylidene fluoride membrane by electroblotting. Immunodetection of proteins was performed using a polyclonal antibody to the carboxy terminus of translocase (*8*) as the primary antibody, and a commercial anti-rabbit IgG conjugated to alkaline phosphatase as the secondary antibody, and visualized.

MTS-Mediated Inhibition Assays. Inhibition of the transport activity of single-cysteine mutants of *tlc1* by the MTS compounds was determined using the standard ATP transport assay with the following modifications. MTSES, MTSET, or MTSEA was added to the KP_i-diluted cell suspension to give a survey concentration of 1.0, 2.5, or 0.8 mM, respectively, or as indicated in the text, and incubated for 10 min at room temperature before the addition of [α-^{32}P]ATP (50 μM, 1−2 μCi/mL). Sensitivity to these reagents was expressed by comparison of the transport ability of treated versus untreated cells. Dry MTS compounds were dissolved in water immediately prior to addition to the cells to be treated because of their instability in aqueous solution.

Preparation of Inside-Out Vesicles. The method for the preparation of inside-out (ISO) vesicles was adapted from ref *29*. Single-cysteine-containing translocase mutants were grown (150−500 mL), induced with IPTG, washed once in 250 mM sucrose, and suspended in 3 mL of room-temperature 250 mM sucrose with 1 mM KP_i. Because the *R. prowazekii* translocase transports ATP with an obligate exchange mechanism, it was necessary to load ISO vesicles with ATP by including 20 mM ATP in the suspension buffer. Cells were lysed using a French pressure cell once at 8000 psi followed by the addition of DNase I (100 μg) for 5 min. Unbroken cells and other debris were removed by centrifugation; ISO vesicles were collected by ultracentrifugation, and the ISO pellet was suspended in 500 μL of 250 mM sucrose. The ISO vesicles were passed over an AG 1-X-Cl anion exchange resin to remove extracellular ATP. The ISO suspension was diluted to an OD_{600} of 0.25 for ATP transport and MTS inhibition assays as described above. The extent of nonspecific binding of ATP to the cytoplasmic surface of the membrane was large, unlike that for the surface of the whole cells, and this level of binding had to be subtracted to properly calculate the level of inhibition (unloaded, control ISO vesicles served as the background).

ATP Protection Assays. Modifications to the standard filtration assay allowed the treated cells to be washed free of ATP and MTS reagents and transport-labeled ATP while on the filter. ATP was added to cells at a final concentration of 1 mM (control cells were treated with buffer alone) followed immediately by MTSES, MTSET, or MTSEA at 500, 100, or 50 μM, respectively. After incubation for 4 min at room temperature, 100 μL of cells was filtered under low vacuum to remove the protective ATP and the unreacted MTS compound. The filtered cells were washed with 5 mL of KP_i under low vacuum without drying the cells. Two milliliters of 50 μM [α-^{32}P]ATP (10 μCi/mL) was applied to the cells on the filter and incubated for 5 min without vacuum. Full vacuum was then applied and the filter washed with 20 mL of KP_i. The central cell material was cut away from the remainder of the filter with a 1.7 cm cork borer to eliminate the radioactivity that might be trapped at the filter's edge.

RESULTS

Cysteine-Scanning Mutagenesis of TM VIII. The previously described cysteine-less mutant of the *R. prowazekii* ATP/ADP translocase with an affinity for ATP indistinguishable from that of the wild type (*3*) was subjected to site-directed mutagenesis to engineer a library of single-cysteine substitution mutants from Q321 to L341 (the putative span of TM VIII) (Figure 1). Although previous studies have shown there to be 12 transmembrane domains (*5*), there remains an absence of empirical data that precisely identify the amino acids at the cytoplasmic and periplasmic boundaries of TM VIII. The amino acid residues of TM VIII we selected were predicted by the MEMSAT, HMMTOP, and TMHMM programs (*30*−*32*). It seems reasonable that the two charged amino acids, R342 and K343, would represent the junction of TM VIII and cytosolic loop 9, leaving L341 as the last amino acid residue of the transmembrane segment. The remaining residues of TM VIII were extrapolated from the TM prediction program data. All mutant constructs were sequenced to confirm the presence of the cysteine substitution and the absence of any PCR-introduced secondary mutations, expressed in the BL21(DE3) strain of *E. coli*, and assayed for their ability to transport ATP.

The results presented in Figure 2 demonstrate that all but two of the mutants exhibited a significant amount of transport activity (other mutants displayed activity that was between 25 and 226% of that of the cysteine-less parent). Substitutions at positions Q323 and G327 resulted in near total ablation of transport, indicating that cysteine substitution is not tolerated at these positions and may suggest that the conformation of the protein near these residues is critical to translocase activity. Interestingly, a cysteine substitution at position F324 resulted in a mutant translocase that had a transport activity that was >200% of that of the cysteine-less parent protein. Activity much greater than that of the wild type has also been observed in some translocase mutants where lysine residues in the hydrophilic loops were mutated to cysteine (*7*). Although the molecular basis of such increased activity is unknown, it is clear that the evolution of the translocase protein was not driven by only the magnitude of the extent of ATP uptake. Immunoblot analysis confirmed that neither the increased activity in the F324C construct nor the inactivity of the two nontransporters, Q323C and G327C, could be attributed to aberrant protein expression (data not shown). This analysis was not necessary for the other mutants since sufficient activity, not maximal expression, was required for our study.

Reactivity of Cysteine Residues within TM VIII with Hydrophilic Thiol Reactive Compounds. The goal of this analysis was to determine if any portion of TM VIII contributes to the structure of the water-filled channel through which the substrate (ATP) is translocated into the cell. Any time a cysteine residue has replaced an amino acid that is normally exposed to an aqueous channel, MTSES, MTSET, or MTSEA should have access to the thiol group on the cysteine and react with it to form a covalently linked, cysteine−MTS adduct. Conversely, any positions in TM VIII

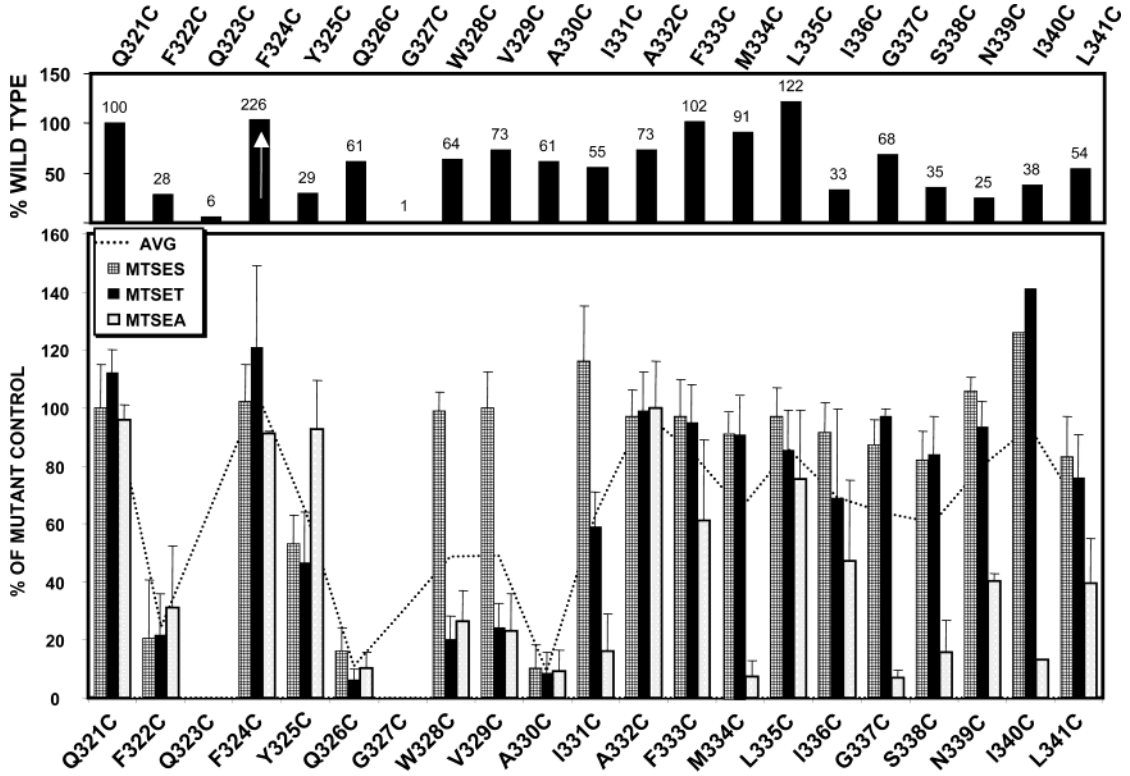

FIGURE 2: (Top) Ability of single-cysteine mutants of TM VIII to transport ATP. Mutants are designated by the single-letter amino acid abbreviation for the targeted residue, followed by the sequence position number in the ATP/ADP translocase, and a second letter indicating the replacement. The arrow on the data bar for F324C denotes that activity exceeds the scale that is depicted. (Bottom) Effect of MTSET, MTSES, and MTSEA on the ability of single-cysteine mutants of TM VIII to transport ATP. AVG (•••) refers to the mean effect of all three reagents.

that are sequestered from the aqueous channel should be virtually inaccessible to MTSES and MTSET and may be variably reactive with MTSEA [that with a p*K* of 8.5 can become un-ionized (*33*)].

Five patterns of inhibition of ATP transport by the MTS reagents (added to the periplasmic side at excess concentrations) were observed in the 19 mutants that retained ATP transport (Figure 2). (i) ATP transport was not inhibited by any of the three MTS reagents in mutants Q321C, F324C, A332C, and L335C and was only marginally inhibited in F333C (percentage of control was $64 \pm 28\%$, $n = 10$). This would suggest either that modification of these cysteine residues could be tolerated and transport still occurred or that these residues were inaccessible to all three reagents. (ii) Also in an all or none fashion, the activity of translocase mutants F322C, Q326C, and A330C was markedly inhibited by all three reagents. This indicates that these three residues were very accessible to hydrophilic reagents and that these mutants were unable to transport ATP after the cysteine modification. (iii) ATP transport was inhibited by MTSEA, but not by MTSES or MTSET, in the largest group of mutants (M334C, I336C, G337C, S338C, N339C, I340C, and I341C). While this group of mutants was clearly sensitive to the formation of an MTS adduct, these residues are likely only accessible to MTSEA which has some hydrophobic properties (*33*). (iv) Transport activity was inhibited by the positively charged MTSET and MTSEA, but high concentrations of the negatively charged MTSES were required for inhibition in mutants W328C, V329C, and I331C. This indicated that the negatively charged MTSES has poor access to cysteines at these positions relative to the positively charged reagents. (v) Finally, ATP transport by mutant Y325C was unaffected by MTSEA but was inhibited 52 and 60% in the presence of MTSES and MTSET, respectively. Perhaps this is because the MTSEA adduct is the smallest of the three adducts (*17*) and, in this case, the only one that does not result in steric hindrance.

Mutants W328C, V329C, and I331C Exhibit Sensitivity to High Concentrations of MTSES, and the Sensitivity of the Mutant W328C Is Influenced by the Concurrent Transport of ATP. The data presented above demonstrate that ATP transport by mutants W328C, V329C, and I331C was inhibited by MTSET and MTSEA, but not by MTSES at the usual survey concentration (Figure 2). We employed a very high concentration of MTSES (50 mM) to attempt to increase its access to these residues [MTSES is the least reactive of the three reagents (*24*)]. As a control to ensure that exposure to high concentrations of MTS compounds does not result in nonspecific inhibition, the cysteine-less parent was exposed to 50 mM MTSES and displayed unaltered levels of transport activity. The results presented in Figure 3 demonstrate that the mutant W328C, as well as mutants V329C and I331C (data not shown), was inhibited by 50 mM MTSES and by 2.5 mM MTSET, but not at 1 mM MTSES. These effects of high concentrations were not general phenomena since two mutants (Q321C and F324C), which were not inhibited by any of the three MTS reagents at the survey concentrations, remained uninhibited when assayed at 50 mM MTSES and MTSET (data not shown). Although MTSEA inhibited mutants W328C, V329C, and I331C at the survey concentration of 0.8 mM, a higher minimum concentration of MTSEA was required to inhibit

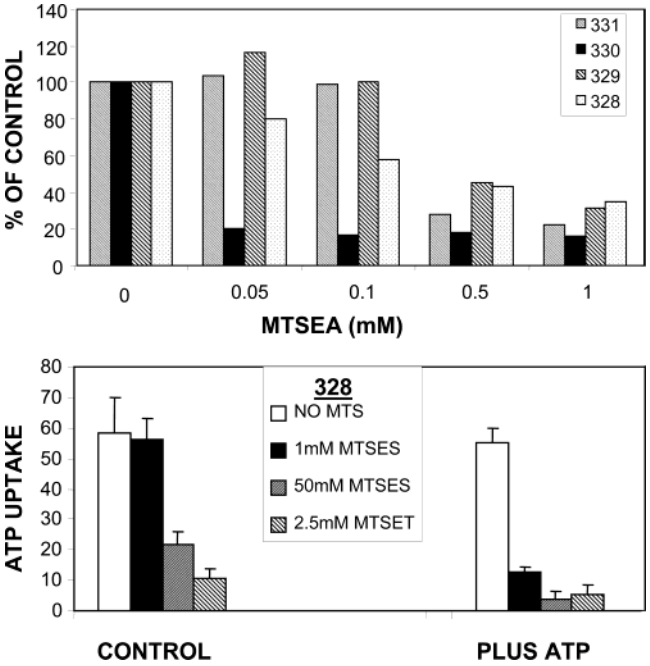

FIGURE 3: (Top) Decreased susceptibility to MTSEA in mutants W328C, V329C, and I331C relative to that of A330C. (Bottom) ATP increases the sensitivity of inhibition by MTSES in mutant W328C.

these three mutants than to inhibit the adjacent mutant A330C, a mutant that was fully inhibited at 0.05 mM (Figure 3).

We also considered that the conformation of the channel might change during translocation of the substrate and increase the accessibility of MTSES in mutants W328C, V329C, and I331C. Therefore, these mutants were exposed to 1 and 50 mM MTSES in the presence or absence of 50 μM ATP [a concentration that would stimulate transport, but would not saturate the translocation pathway since the K_m $\sim$ 100 μM (*3*, *9*)]. Interestingly, when a substrate was present, W328C was dramatically inhibited by 1 mM MTSES (Figure 3). Mutants V329C and I331C were not inhibited by 1 mM MTSES even in the presence of a substrate (data not shown).

M334C, a Mutation of a Cytoplasmic-Proximal Residue, Demonstrates Sensitivity when It Is Exposed to Elevated Concentrations of MTSES and MTSET in the Presence of 50 μM ATP. It should be noted that the six mutants that were inhibited by MTSES and MTSET were all located in the periplasmic-proximal half of TM VIII (Q321−I331, Figure 2). Three mutants (M334C, G337C, and I340C) located in the cytoplasmic-proximal half of TM VIII (A332−L341) were inhibited by MTSEA, but none of the mutants in the cytoplasmic-proximal half were markedly inhibited by the survey concentrations of MTSES or MTSET. Considering the experiments with W328C described above, we postulated that a high MTS concentration and substrate translocation might also expose a cytoplasmic-proximal residue to MTSES and MTSET. Therefore, the mutant M334C that was readily inhibited by MTSEA was assayed with MTSES and MTSET at 50 mM with and without 50 μM ATP. The results presented in Figure 4 demonstrate that an increase in the MTSES and MTSET concentrations resulted in an increased level of inhibition, and the combination of high concentrations in the presence of 50 μM ATP resulted in a further

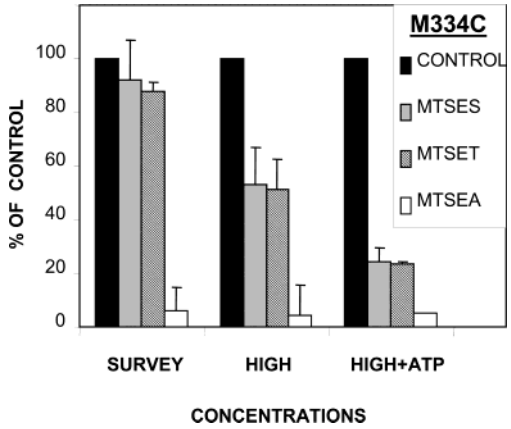

FIGURE 4: Mutant M334C is sensitive to high MTS reagent concentrations, and this sensitivity is greater when ATP is being transported. MTSES, MTSET, and MTSEA were present at the survey concentrations of 1, 2.5, and 0.08 mM, respectively, or at the high concentration of 50 mM. ATP was at a concentration of 50 μM.

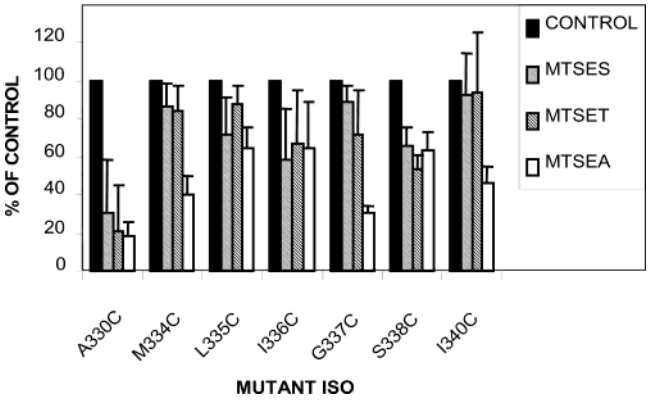

FIGURE 5: Effect of MTS reagents on ATP transport in ISO vesicles.

increase in the extent of transport inhibition. However, high levels of inhibition, comparable to those seen in the periplasmic-proximal mutants, were never observed in the cytoplasmic-proximal mutant.

Whole Cells and Inside-Out (ISO) Vesicles Show No Difference in Susceptibility to MTS-Induced Inhibition of Transport. It was possible that the observed differences in the extent of MTSES and MTSET susceptibility between the two halves of TM VIII were a consequence of limited exposure of the cytosolic-proximal residues, possibly because MTS reagents added to the outside of the cell are precluded for some reason from traveling past the halfway point of the translocation pathway. To test this hypothesis, ISO vesicles, where the reagents could be added to the cytoplasmic side, were assayed to determine whether mutants with cysteines at positions 334−338 and 340 would now be susceptible to the hydrophilic reagents. As a control, the readily inhibitable mutant A330C with a cysteine substitution located near the middle of TM VIII was shown to be sensitive to all three reagents when added from the cytoplasmic side (or periplasmic as shown above) of the membrane (Figure 5). These cytoplasmic-proximal mutants were inhibited by MTSES and MTSET added to ISO vesicles at approximately the same low extent observed in whole cells (Figure 5). The three mutants with cysteines at positions 334, 337, and 340 retained the marked MTSEA sensitivity in ISO vesicles that they displayed in whole cells (Figure 5).

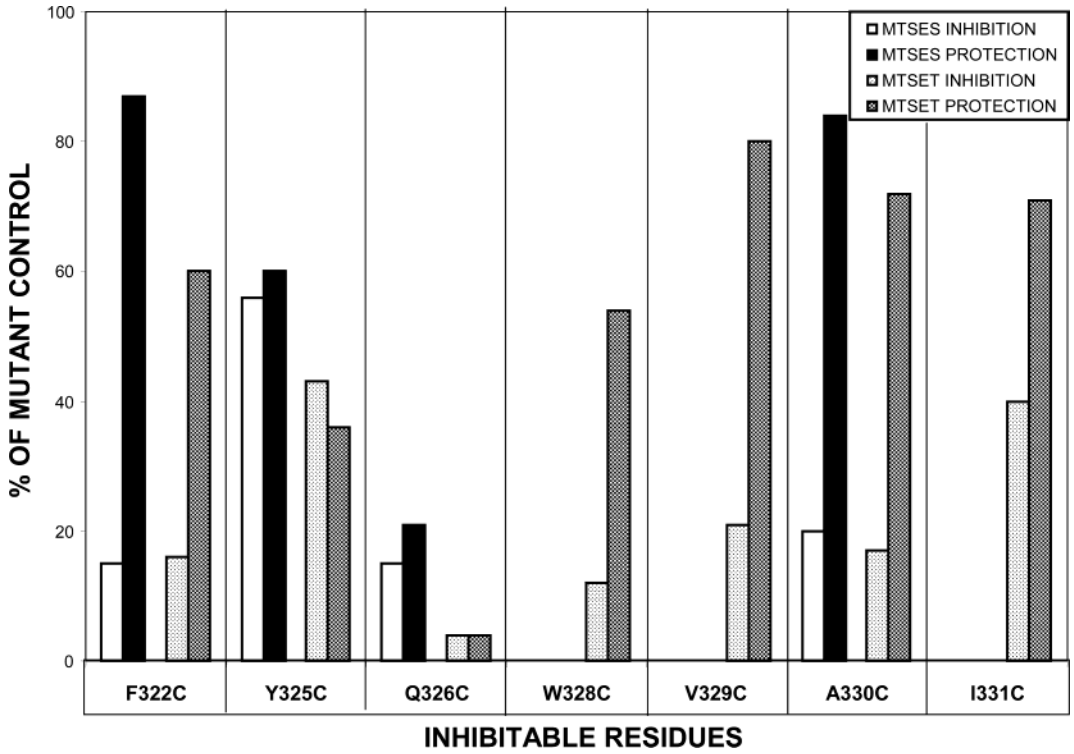

FIGURE 6: Protection from MTS reagent inhibition by ATP. Only those mutants that were inhibited could be tested for protection so that mutants not inhibited by one of the two reagents have empty columns. Mutants were inhibited by MTSET (100 μM), MTSET, and MTSES (500 μM).

Preincubation with 1 mM ATP Protects MTS-Sensitive Mutants from Inhibition. There were seven mutants of TM VIII in the periplasmic-proximal half of TM VIII that were very sensitive to modification by one or both of the MTSES and MTSET reagents (F322C, Y325C, Q326C, W328C, V329C, A330C, and I331C). These residues were, therefore, hypothesized to be exposed to the aqueous channel through which ATP is transported. We postulate that filling the translocation pathway with the substrate, ATP, before and during MTS exposure could, under the appropriate steric constraints, block access of MTSES and MTSET to the cysteine residue and result in protection from thiol modification. To have a ratio of ATP to MTS reagent in the putative channel that would be conducive to protection in these experiments, substrate was added to the cells at a concentration 10 times greater than the K_m, and the lowest concentration of the MTS reagent that gave full inhibition (under nonprotective conditions) was used. Controls were performed with the same protocol either without 1 mM ATP (maximal inhibition) or without MTS reagent (100% transport). Five of the seven sensitive mutants were protected (F322C, W328C, V329C, A330C, and I331C) (Figure 6). Protection was substrate-specific since it was afforded by ATP or ADP and not by related compounds (AMP and GTP) that are not substrates for translocase (*9*) (data not shown). Y325C and Q326C were inhibited by MTSES and MTSET but were not protected, perhaps because the channel is wider where it is adjacent to these residues.

DISCUSSION

Studies of membrane proteins that are poorly expressed and typically recalcitrant to X-ray crystallographic analysis necessitate the use of genetic and alternative biochemical analyses to provide indirect information about three-dimensional structures. This problem is compounded in studies of membrane proteins in *R. prowazekii* that has no well-developed genetic system and must be grown within the cytoplasm of a eukaryotic host cell. Therefore, to gain insight into the nature of the translocation pathway, 21 single-cysteine mutants in TM VIII were constructed, sequenced, expressed in *E. coli*, and tested for the effects of mutagenesis, hydrophilic thiol reactive compounds, and substrate protection.

Fortunately, only two (Q323C and G327C) of the 21 mutants were unable to transport ATP at a rate suitable for further analysis. This suggests considerable permissiveness in the structure−function relationships of TM VIII. Alignment of the sequences of nine translocases from various organisms, all of which displayed an obligate exchange mode of transport with ATP or ADP as a substrate, shows that only F322 and G327 are invariant residues in TM VIII (Figure 7). Furthermore, alignment of all similar transport proteins, irrespective of mechanism or substrate, shows only G327 is invariant. The mutant G327C was unable to transport ATP, but the mutant F322C had low but measurable activity (28%). It is surprising that the mutant with cysteine substituted at position Q323 was unable to transport ATP since both chlamydial and plastidic translocases have serine at this position (Figure 7). The permissiveness of TM VIII is also indicated by the fact that the chlamydiae translocases have a serine inserted between (rickettsial) positions 329 and 330 that would change the facial exposure of a helical molecule distal to the insertion (Figure 7).

The helical model of transmembrane helix VIII depicted in Figure 8 highlights the amino acid positions that were susceptible to MTS-mediated inhibition of transport. Clearly,

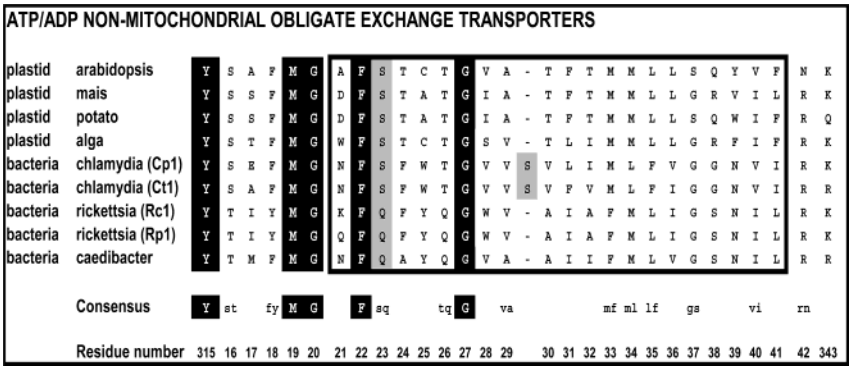

FIGURE 7: Alignment of the protein sequence near TM VIII of rickettsial-like ATP/ADP translocases. The putative 21 residues of TM VIII are boxed. The 100% conserved residues are shown as white letters on a black background. Consensus residues with two variations are shown in lowercase. Residues that are shaded with gray are discussed in the text.

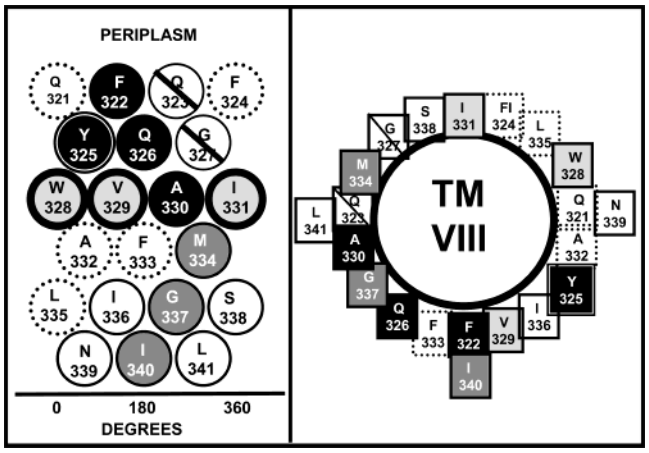

FIGURE 8: Helical web and wheel of TM VIII. Residues are arranged with α-helical, 100° spacing. Three mutants inhibited by all three MTS reagents are shown in white on a black background. One mutant inhibited by all MTS reagents except MTSEA is shown in white on a black background with a white border. Three mutants with low sensitivity to MTSES are shown in black on a gray background with a black border. Three mutants markedly inhibited by only MTSEA are shown in white on a gray background. Four mutants modestly inhibited by only MTSEA are shown in black on a white background with a solid border. Five mutants not inhibited are shown in black on a white background with a dotted border. Two mutants with no uptake are shown with strike-out lines.

this does not support a simple model of a single water-accessible face with the other faces buried in a lipid or proteinaceous environment and a periodicity of 3.6 amino acids per turn. The most prominent feature of this model is the marked difference between the periplasmic- and cytoplasmic-proximal mutants with the former being highly inhibited by MTSES and MTSET and the latter being poorly inhibited. It is also noteworthy that the three most highly MTSES- and MTSET-inhibited mutants in the periplasmic-proximal half (F322C, Q326C, and A330C) and the three most MTSEA inhabitable of the cytoplasmic-proximal mutants (M334C, G337C, and I340C) are aligned on a single face with the standard α-helical periodicity (Figure 8). ATP transport in mutants M334C, G337C, and I340C was strikingly inhibited by MTSEA, the smallest of the three MTS reagents, but not appreciably inhibited by MTSES or MTSET in either whole cells or ISO vesicles. This strongly suggests that residues in the cytoplasmic-proximal half of TM VIII are not lining a translocation pathway that is large enough to accommodate ATP. The strong inhibition by MTSEA demonstrates that these three residues inhibit translocation when converted to the MTSEA adduct. Therefore, it follows that the larger, similarly charged MTSET must be inaccessible, since its adduct, if formed, would have inhibited transport. This suggests that the un-ionized form of MTSEA gains access to these residues by partitioning through the lipid matrix (*33*). Alternatively, since the thiol group of any cysteine residues must be ionized in an aqueous environment to react with MTSEA and we observed that very high concentrations of MTSET and MTSES can inhibit transport in the cytoplasmic-proximal mutant M334, but not in the cysteine-less parent, an alternative view can be hypothesized. To wit, there is a small-diameter, water-filled pathway from both the periplasmic and cytoplasmic sides of the membrane to these cytoplasmic-proximal residues through which the MTS reagents can travel, but the much larger ATP cannot. This small diameter favors the movement of the smallest reagent MTSEA over the larger MTSES and MTSET so that enormous concentrations of the latter two are required for them to reach the residues in the cytoplasmic-proximal half of TM VIII.

Mutants in the central part of TM VIII located in the three residues adjacent to A330 were inhibited by the survey concentrations of MTSET and MTSEA, but not by MTSES. They form a continuous stretch of four residues (W328C, V329C, A330C, and I331C) that were all accessible to hydrophilic MTS compounds, and all were protected by the presence of substrate in the translocation pathway (Figure 6). This suggests that while all four residues are exposed to the aqueous channel, only one of the four, A330, is directly in the channel. The other three residues may be in a "blind bayou" that can communicate with the channel, but in a restricted manner.

Five of the MTS-sensitive sites were protected from MTS-mediated inhibition of transport when they were co-incubated with 1 mM ATP (producing a flux of 90% of V_{max}). It is clearly indicated that these sites line, or at least communicate with, the translocation pathway. Whether they are also substrate binding sites is unknown. It should be noted that the ATP typically present in the cytosol at ~5 mM is not sufficient to provide protection, and this ATP must not have access to the residues in the translocation pathway in the absence of the exchange brought about by the addition of extracellular ATP. This indicates that the region of the translocation pathway formed by the periplasmic-proximal residues, the region where protection occurs, is empty of substrate until exchange occurs. This is in accord with the

fact that there is no unidirectional movement of ATP either into or out of the cell (*9*).

Regardless of the subtle nuances observed here, our data suggest that (i) at least the periplasmic-proximal half of TM VIII makes up a portion of the translocation pathway for ATP as evidenced by the accessibility of MTS reagents and the substrate-specific protection afforded and (ii) the translocation pathway for ATP is a dynamic structure in which the residues are not locked in one conformation. The latter is dramatically seen with the mutant W328C where the translocation of substrate at only 33% of V_{max} makes this formerly inaccessible residue accessible to 1 mM MTSES (Figure 5). A model, in which the two halves of TM VIII and the adjacent transmembrane regions are packed differently, can provide a structure in which the two halves of TM VIII have differential access to the translocation pathway. It is also hypothesized that this packing of the transmembrane domains allows access of consecutive residues 328−331 to the translocation pathway. This study serves as the model for the resolution of the relationship between the translocation pathway and the remaining 11 transmembrane regions so that the assemblage of the remaining pieces of this puzzle will provide insight into the structural and functional properties of the ATP/ADP translocase.

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