Cysteine-Scanning Mutagenesis and Thiol Modification of the *Rickettsia prowazekii* ATP/ADP Translocase: Evidence That Transmembrane Regions I and II, but Not III, Are Structural Components of the Aqueous Translocation Channel†

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ABSTRACT: The contribution of transmembrane regions I, II, and III of the *Rickettsia prowazekii* ATP/ADP translocase to the structure of the putative water-filled ATP translocation channel was evaluated from the accessibility of hydrophilic, thiol-reactive, methanethiosulfonate reagents to a library of 68 independent cysteine-substitution mutants heterologously expressed in *Escherichia coli*. The MTS reagents used were MTSES (negatively charged) and MTSET and MTSEA (both positively charged). Mutants F036C, Y042C, and R046C (TM I), K066C and P072C (TM II), and F101C, F105C, F108C, Y113C, and P114C (TM III) had no assayable transport activity, indicating that cysteine substitution at these positions may not be tolerated. All three MTS reagents inhibit the transport of ATP in mutants of TM I (L039C, S043C, S047C, I048C) and TM II (S061C, S063C, T067C, I069C, V070C, A074C). Further, these residues appear to cluster along a single face of the transmembrane domain. Preexposure of MTS-reactive mutants S047C (TM I) and T067C (TM II) to high levels of ATP resulted in protection from MTS-mediated inhibition. This indicated that both TM I and TM II make major contributions to the structure of an aqueous ATP translocation pathway. Finally, on the basis of the lack of accessibility of charged MTS reagents to the thiol groups in mutants of TM III, it appears that TM III is not exposed to the ATP translocation channel. Cysteine substitution of residues constituting a highly conserved "phenylalanine face" in TM III resulted in ablation of ATP transport activity. Further, substituting these phenylalanine residues for either isoleucine or tyrosine also resulted in much lower transport activity, indicating that some property of phenylalanine at these positions that is not shared by cysteine, isoleucine, or tyrosine is critical to translocase activity.

Rickettsia prowazekii, the agent of epidemic typhus in humans, is an obligate intracellular alpha proteobacteria with a Gram-negative architecture that grows only within eukaryotic host cell cytoplasm, unbound by any host-derived membrane structure (for reviews see refs *1* and *2*). As a consequence of sequestration in a cytosolic niche, this unusual bacterium has evolved transporters that allow it access to the bevy of metabolic products that are available in the host cell's cytoplasm. In contrast, bacteria that live in the extracellular environment are unable to transport the typical large, charged cytosolic metabolites, such as ATP, which are rarely available in the extracellular niche.

The ATP/ADP translocase of *R. prowazekii* is an obligate exchange antiporter that facilitates the uptake of host cell ATP in exchange for rickettsial ADP as a source of energy, not adenylate. The transport of ATP/ADP by the *R. prowazekii* translocase (Tlc1)[1] has been characterized in both purified rickettsiae and *Escherichia coli* that express a plasmid-borne, rickettsial *tlc1* gene in trans (*3*−*11*). The ATP/ADP translocase is at present the sole member of a distinct transport protein family in the classification scheme of Saier (*12*).

The problems typically associated with the purification of membrane proteins in a form suitable for X-ray crystallographic analysis have necessitated the use of genetic and alternative biochemical approaches for studies of translocase topology. Our previous work has utilized dual *pho-lac* reporters fused to the *tlc1* gene to provide evidence that the ATP/ADP translocase has 12 transmembrane regions and that the 7 odd-numbered hydrophilic loops are on the cytoplasmic side of the membrane and the 6 even-numbered loops are on the periplasmic side (Figure 1) (*4*). ATP/ADP translocases with similar function and amino acid sequences have now

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[1] Abbreviations: TM I, II, and III, transmembrane regions I, II, and III, respectively; 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; ISO, inside-out vesicles; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; KP_i, potassium phosphate buffer; $LBAp^{100}$, Luria−Bertani media supplemented with 100 μg/mL ampicillin; $TBAp^{100}$, Terrific broth media supplemented with 100 μg/mL ampicillin; Tlc1, the *Rickettsia prowazekii* ATP/ADP translocase.

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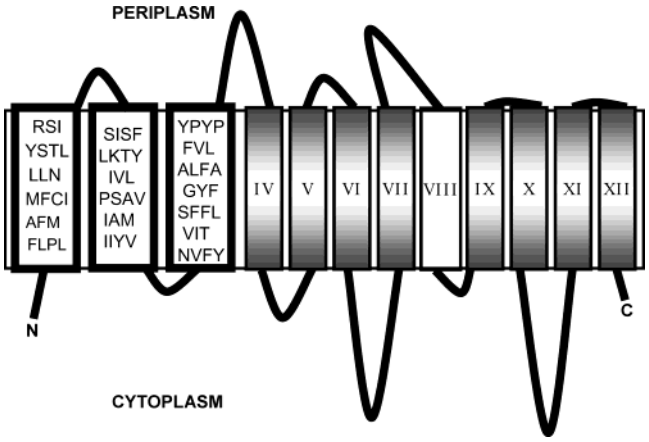

FIGURE 1: Model of the rickettsial ATP/ADP translocase. The hydrophilic loops connecting the 12 transmembrane regions have lengths proportional to the number of residues in each loop. The N- and the C-termini and their periplasmic/cytoplasmic orientation are indicated. TM regions in gray have not yet been analyzed. Analysis of TM VIII (in white) was previously reported. The amino acid residues constituting each of the first three TM domains are depicted.

been identified in all obligate intracellular bacteria investigated to date including the human pathogens, rickettsiae and chlamydiae, and the protistic endosymbionts, holospora and caedibacter (*13*−*15*). Curiously, this translocase is present in the plastids of plants ranging from land plants to red algae despite the fact that a translocase analogue is conspicuously absent in the putative ancestor of these organelles, namely, cyanobacteria (*16*, *17*). In addition, although rickettsiae are the closest related bacteria to the mitochondria on an evolutionary scale, the mitochondrial ADP/ATP exchanger shows no sequence homology and little structural similarity with the rickettsial ATP/ADP translocase. Further, their functions are reversed; i.e., the parasite takes ATP from the host cell whereas the mitochondrion provides ATP to the host cell (*16*, *18*).

In the present study, we determined whether transmembrane regions I, II, and III (TM I, TM II, and TM III) form part of the structure of an aqueous channel that must exist to provide the medium through which ATP and ADP are translocated across the membrane. To this end, a previously constructed, cysteine-less mutant of the *R. prowazekii tlc1* gene that has been resynthesized to possess an *E. coli*-like codon usage and multiple unique restriction endonuclease recognition sites cloned in a pT7-5 expression vector (without any affinity tags or other modifications) (*4*) was subjected to site-directed mutagenesis, resulting in a library of mutants in TM I, TM II, and TM III, each containing a single-cysteine substitution. These single-cysteine-containing Tlc1 mutants were then assayed for ATP uptake by accumulation of [α-^{32}P]-ATP. Single-cysteine-containing mutants that exhibited measurable transport activity were subsequently exposed to specific, highly thiol-reactive reagents to determine if these MTS reagents have access to residues in any of the first three TM of Tlc1. The sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) and [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) reagents are charged and cannot enter the lipid domain of the membrane, whereas 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA), in contrast, can exist in either a charged or uncharged form. Therefore, residues inhibited by charged MTS compounds would be candidates to line the water-filled channel through which ATP is translocated. Mutants with MTS-accessible residues were also tested to determine whether the presence of substrate (i.e., ATP) in the translocation pathway would protect these residues from the hydrophilic reagents. We have successfully employed these strategies to determine that TM VIII of the *R. prowazekii* translocase possesses residues that are exposed to the aqueous environment and are, therefore, candidates to form the water-filled ATP translocation channel (*19*). Strategies similar to this have been employed to examine the membrane topology of transport proteins in other families of transport proteins (*20*−*30*). It is important to note that, regardless of the technique utilized, all of these studies must assume that the cysteine substitution does not change the native conformation of the protein.

MATERIALS AND METHODS

Growth Conditions and Reagents Used in This Study. Bacteria were routinely grown in Luria−Bertani (LB) broth or TB media supplemented with 100 μg/mL ampicillin (LBAp^{100} or TBAp^{100}) at 37 °C with aeration. The MTSES, MTSET, and MTSEA compounds were purchased from Toronto Research Chemicals Inc. (North York, Canada) and dissolved for use at the beginning of each experiment.

Single-Cysteine Substitution Mutant Construction. The site-directed mutagenesis was performed using pMA613ΔC (pT7-5 backbone) as a template. Plasmid pMA613ΔC is a pMA613 derivative (containing the *R. prowazekii tlc1* gene) (*4*) that is a C037A C085A double mutant. This vector expresses a cysteine-less *tlc1* gene that has been resynthesized to introduce an *E. coli*-like codon usage and multiple, unique restriction endonuclease recognition sites to facilitate mutagenesis (*4*). The mutagenesis was performed by using either overlap-extension PCR (*31*) or megaprimer PCR (*32*) to produce a bank of single-cysteine substitution mutants representing every amino acid residue of TM I−III. In all cases, unique restriction endonuclease recognition sites flanking a TM allowed for the excision and mutagenesis of small fragments of Tlc1-encoding DNA. Mutagenesis of shorter regions of DNA decreases the risk of introducing secondary mutations generated by polymerase error. Further, confirming the presence of the desired mutation requires sequencing of short regions only as opposed to sequencing the entire gene. Mutagenized constructs were transformed into a chemically competent DH5α strain of *E. coli* [F$^-$ 080d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* 1- *thi-1 gyrA96 relA1*] and selected on LBAp^{100} agar plates. Once the presence of the desired mutation (and a lack of undesired, PCR-induced secondary mutation) was confirmed by sequencing, plasmids were introduced into a chemically competent C41 strain of *E. coli* [a derivative of BL21(DE3) (*33*)] allowing for IPTG-induced expression from the T7 promoter.

Assay of Single-Cysteine Substitution Mutants for MTS-Mediated Inhibition of ATP Transport. All of the single-cysteine substitution mutants constructed in this study were tested for their ability to transport ATP after exposure to hydrophilic MTS reagents as previously described (*19*). Briefly, overnight cultures of *E. coli* C41 harboring plasmids expressing either the parent (synthetic, cysteine-less translocase) or single-cysteine substitution mutants grown in either

LBAp[100] or TBAp[100] were subcultured into fresh media and grown to an OD_{600} of 0.4, and IPTG was added (1 mM final concentration). After 1 h of induction, a 30-fold excess of 50 mM potassium phosphate buffer, pH 7.5 (KP_i), was added to wash the bacteria followed by centrifugation and suspension of the cells to their original volume in KP_i (all operations on ice). Bacteria were then incubated with and without MTSES, MTSET, or MTSEA (10 mM final concentration) for 10 min at 34 °C followed by the addition of [α-^{32}P]ATP (final concentration of 50 μM, 1−2 μCi mL^{-1}). ATP transport was determined at 9 and 10 min by filtration on Millipore HA filters as previously described (*19*, *34*). At the end of the uptake assay bacteria were filtered and washed, and the radioactivity on the filter was measured to determine the extent of ATP uptake. Sensitivity to hydrophilic MTS reagents was expressed by comparison of the transport ability of treated vs untreated cells. Dry MTS compounds were dissolved in KP_i immediately prior to addition to the cells to be treated because of their instability in aqueous solution.

The C41 expression strain harboring the Cys-less parent plasmid, on average, was nonspecifically inhibited about 10% by these concentrations of MTS reagents. Further, the plasmid-less C41 expression strain displayed about 8% nonspecific ^{32}P association compared to the Cys-less parent. Because of this nonspecific inhibition of a Cys-less parent and the nonspecific association of ^{32}P with strains not possessing the tlc, to be conservative, we demanded that any mutant must exhibit at least 50% inhibition to be categorized as "inhibitable". All mutants were assayed for MTS inhibition (Figure 2). However, low transporters that generated data with large standard deviations could not be distinguished from background and were, therefore, not reported. In all cases where the standard deviations were small, the data were reported.

ISO vesicles of selected mutants (see text) were prepared and assayed as previously described (*19*).

ATP Protection Assays. In our previous study of translocase TM VIII it was determined that the presence of excess substrate (i.e., ATP) protected a subset of mutants from MTS-mediated inhibition of transport (*19*). Therefore, mutants of TM I and II that displayed MTS-mediated inhibition of ATP transport were also tested for protection as follows. Inhibitable mutants were grown and washed as described above. Cells were then exposed to the following conditions before the addition of [α-^{32}P]ATP: (i) untreated cells were preincubated in the presence or absence of 1 mM ATP (control conditions); (ii) cells were preincubated in the presence of 0.5 mM MTSES, 0.1 mM MTSET, or 0.05 mM MTSEA (inhibited condition); and (iii) cells were preincubated with 1 mM ATP for 2 min followed by the addition of the above concentration of MTS reagent (protected condition). After 10 min of incubation at 34 °C, cysteine was added to a final concentration of 2 mM to inactivate residual MTS reagent (2 mM cysteine does not inhibit ATP transport; data not shown). Uptake assays were then carried out at a final ATP concentration of 1 mM (1−2 μCi/mL [α-^{32}P]ATP) with samples being removed, filtered, and washed after 9 and 10 min.

RESULTS

Cysteine-Scanning Mutagenesis: TM I, TM II, and TM III. Plasmid pMA613ΔC was subjected to site-directed mutagenesis to engineer a library of mutants in which each of the putative, membrane-spanning amino acids of TM I, TM II, and TM III (Figure 1) were replaced, one at a time, with cysteine. We used the MEMSTAT, HMMTOP, and TMHMM programs (*35*−*37*) to predict the amino acids that make up the transmembrane segments of translocase. Previous studies of the topology of Tlc1 by dual-reporter fusion and antibody accessibility methods (*6*, *8*) support these predictions. However, to ensure coverage of the entire TM, the boundaries of each of the first three TM were extended so as to be flanked by charged amino acid residues as follows: TM I, K027−K049; TM II, E060−K083; and TM III, E091−D117. Single-cysteine-containing mutants were constructed by PCR mutagenesis, sequenced, expressed in *E. coli* strain C41, and assayed for ATP uptake.

In each of the three TM characterized in this study, there were residues in which a cysteine substitution resulted in near total ablation of transport activity. It is possible that ablation of ATP transport activity indicates that the cysteine substitution has affected protein function. There are, of course, other possibilities including lack of protein production or improper insertion (caused by the cysteine substitution) that we have not ruled out. However, only mutants with substantial transport activity that can be assayed for MTS-mediated inhibition of ATP transport are useful to this study. Accordingly, we will not comment on the mechanism of inactivation of transport-negative mutants. Again, studies of this nature must presuppose that the mutants with transport activity have retained the native protein conformation. Mutants F036C, Y042C, and R046C (TM I), K066C and P072C (TM II), and F101C, F105C, F108C, Y113C, and P114C (TM III) had no assayable transport activity. The remaining mutants displayed a substantial level of ATP transport that could be reliably measured and characterized with respect to MTS-mediated inhibition of transport activity (Figure 2). It is interesting to note that substitutions at charged residues (R046C and K066C) predicted to reside in a transmembrane segment (indicating exposure to the aqueous environment) were not tolerated, suggesting that these residues may be critical to transport activity. This result confirms our previous study in which every basic amino acid in Tlc1 was replaced by cysteine (*7*).

Reactivity of Cysteine Residues within TM I, TM II, and TM III with Hydrophilic Thiol-Reactive Compounds. Our premise is that if a cysteine residue in a TM region is inhibited by MTSEA and exposed to the aqueous channel, it will be modified by the charged MTS compounds. Formation of covalently linked cysteine−MTS adducts are identified if transport activity is reduced after exposure to the MTS compounds. The converse also holds true in that any positions that are sequestered from the aqueous phase should be inaccessible to the charged MTSES and MTSET reagents and variably accessible to MTSEA that with a p*K* of 8.5 can be un-ionized (*38*).

On the basis of the inhibition patterns exhibited by residues in TM I−III in response to MTS exposure it appears that portions of both TM I and TM II are exposed to the aqueous environment whereas TM III is not (Figure 2). In TM I, three basic patterns are evident (Figure 2, top panel). (i) ATP transport was inhibited in the presence of all three MTS reagents in mutants L039C, S043C, S047C, and I048C, providing evidence that these residues are exposed to an

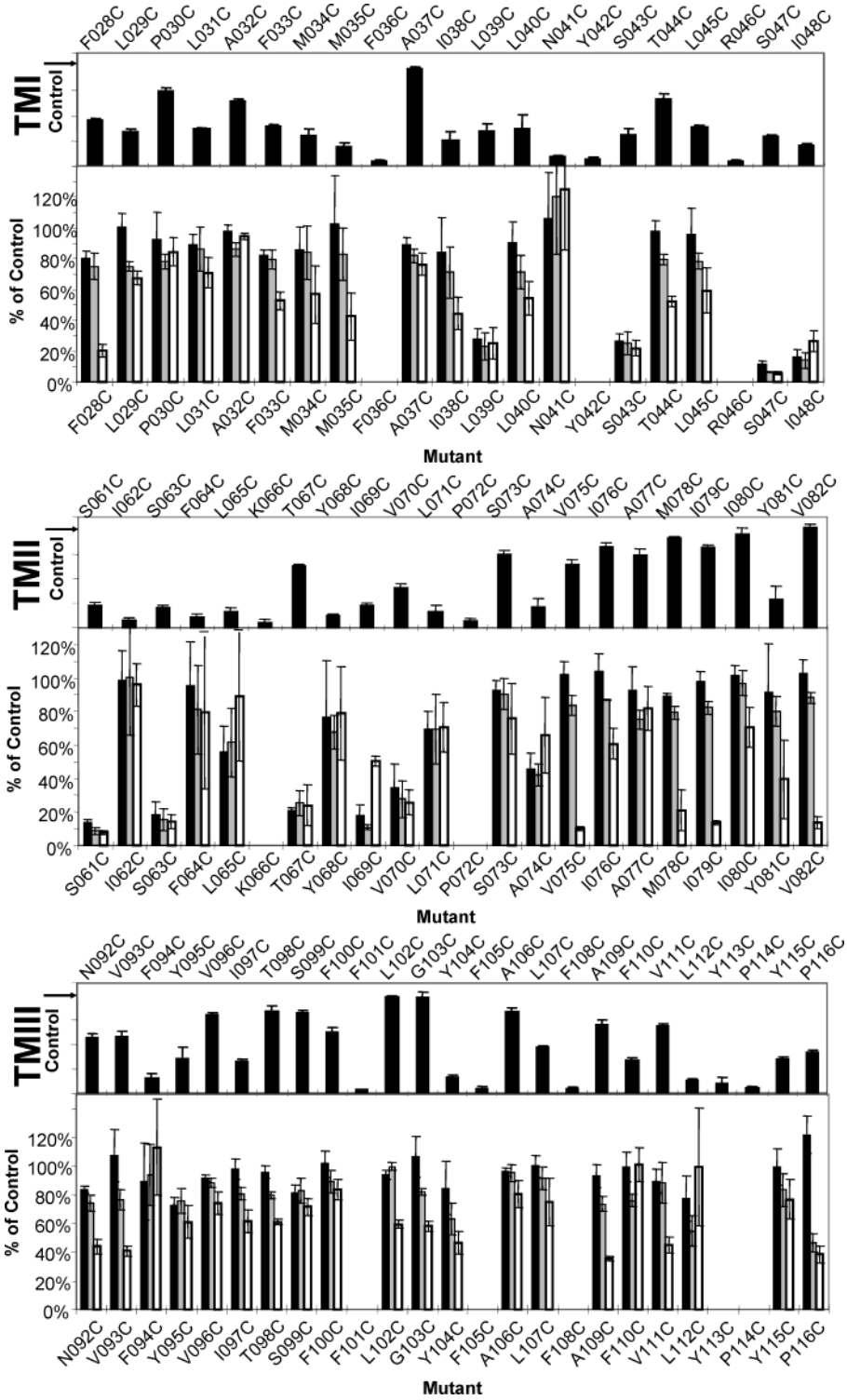

FIGURE 2: Reactivity of cysteine residues within TM I (top panel), TM II (middle panel), and TM III (bottom panel) with hydrophilic, thiol-reactive MTS compounds. The upper portion of each of the three panels shows the ability of single-cysteine-substituted mutants to transport ATP. Mutants are designated 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 cysteine replacement. Activity is expressed as a percent of the cysteine-less parent [about 4 nmol (mg of protein)$^{-1}$ min^{-1} (*4*)] with the arrow denoting 100%. The lower portion of each of the three panels shows the effect of MTSET, MTSES, and MTSEA on the ability of single-cysteine-substituted mutants to transport ATP. Activity is expressed as a percent of control (no MTS reagent) with standard deviations of quadruplicate values from at least two independent experiments. Key: black bars, MTSES; gray bars, MTSET; white bars, MTSEA.

aqueous environment. The wheel diagrams in Figure 3 lend support to this hypothesis. The first three of these residues and R046 (a charged residue that would be predicted to be exposed to the aqueous phase) are clustered along the same side of the helix. Residue I048 is not on this face. However, since I048 is the most periplasmic-proximal residue, it is possible that it is in the periplasm and, therefore, not part of TM I. (ii) ATP transport was not affected by exposure to any of the MTS reagents in mutants L029C, P030C, L031C, A032C, A037C, and N041C, suggesting that these positions

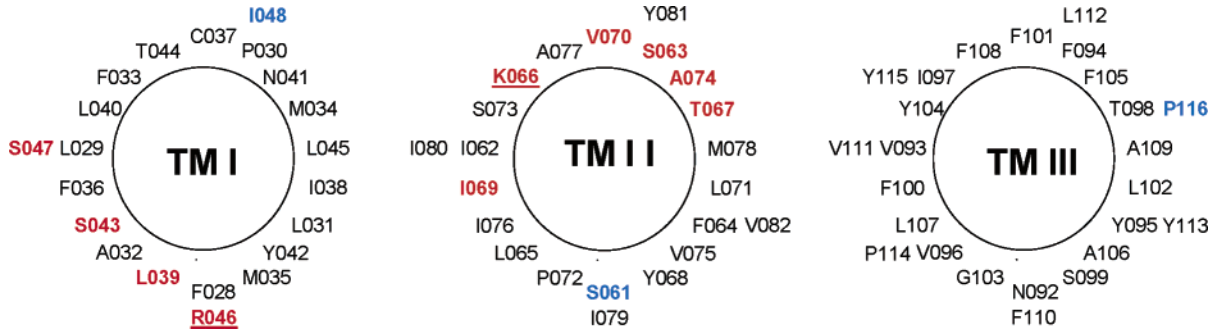

FIGURE 3: Wheel diagrams of TM I, II, and III. Mutants that were inhibited by all three MTS reagents are in red. Mutants R046C in TM I and K066C in TM II are underlined to denote that they had no assayable activity but are colored in red because they are charged residues that cluster on the putative aqueous face of the helix. Mutants that were susceptible to all three MTS reagents but are suspected to reside in the periplasm are in blue (see text).

are not exposed to a water-filled channel or that covalent modifications of cysteine residues by MTS compounds at these positions do not adversely affect ATP transport. (iii) The final group of mutants displayed MTS-mediated inhibition only in the presence of MTSEA (F028C, F033C, M034C, M035C, I038C, L040C, T044C, and L045C). The fact these mutants were inhibited by MTSEA and not MTSES or MTSET indicates that MTS modification is inhibitory to ATP transport and that MTSES and MTSET do not have access to these positions whereas MTSEA (which can become un-ionized) can access these positions by partitioning through the lipid phase.

These three patterns of inhibition were also displayed by TM II (Figure 2, middle panel). (i) Inhibition of six mutants by all three MTS compounds (S061C, S063C, T067C, I069C, V070C, and A074C) indicated that these are residues exposed to an aqueous environment. With the exception of S061, all of these residues are on the same face of the helix (Figure 3). Interestingly, K066, a charged residue likely to be exposed to the aqueous phase, also clusters on this face (Figure 3). Again, S061 is the most periplasmic-proximal residue and may not be part of TM II. (ii) Mutants that were not inhibited by any MTS compound were I062C, F064C, L065C, Y068C, L071C, S073C, I076C, A077C, and I080C, indicating either the lack of aqueous exposure or the failure of the MTS adducts to inhibit ATP transport. (iii) The final group of mutants inhibited by MTSEA only was V075C, M078C, I079C, Y081C, and V082C, indicating their inaccessibility to charged MTS compounds.

TM III displayed a different pattern in that none of the mutants tested were inhibited by all three MTS compounds (Figure 2, bottom panel). Five of the mutants were inhibited only in the presence of MTSEA (N092C, V093C, Y104C, A109C, and V111C), indicating that these mutants can be inhibited by MTS modification but are not accessible to MTSES and MTSET. Mutant P116C is nearest to the periplasm and was inhibited by MTSET and MTSEA but not MTSES. However, because TM III was extended to 25 residues, it is unlikely that P116 is part of TM III. The remainder of the mutants in TM III either displayed no ATP uptake (F101C, F105C, F108C, Y113C, and P114C) or were not inhibited more than 50% by MTSES, MTSET, or MTSEA (F094C, Y098C, V096C, I097C, T098C, S099C, F100C, L102C, G103C, A106C, L107C, F110C, L112C, and Y115C). These results strongly suggest that TM III does not contribute to the structure of the water-filled, substrate translocation channel.

MTS-Mediated Inhibition of ATP Transport by ISO Vesicles Mimics That Seen in Whole Cells. In previous work with TM VIII (*19*) those mutants with cytoplasmic-adjacent residues were assayed as both whole cells and ISO vesicles to determine if addition of MTS reagents to the cytoplasmic side would confirm/extend our results. These ISO vesicles behaved in a manner that was very similar to that of whole cells (*19*). In the present study we selected seven mutants, of which four are shown in Figure 4. Mutants that were inhibited by all three MTS reagents in whole cells (S047C, T067C) were also inhibited in ISO vesicles. Mutants from TM III that were only inhibited by MTSEA in whole cells (A109C, V111C) were only inhibited by MTSEA in ISO vesicles (Figure 4 and data not shown). We also assayed mutants in TM I and II predicted to be adjacent (M078C) or distal (I038C, I079C) to the putative water-filled channel (see Figure 3) that were inhibited by MTSEA, but not MTSES or MTSET, in whole cells. ISO vesicles displayed the same inhibitory profile as whole cells (Figure 4 and data not shown).

Preincubation with 1 mM ATP Is Protective against MTS-Mediated Inhibition. We postulate that filling the channel with an excess of substrate, prior to MTS exposure, could protect MTS-inhibitable mutants from thiol modification. Protection should be favored only in those regions of the channel where steric constraints preclude both ATP and the MTS compound from occupying the same space, and because ATP is added first, these positions are blocked from subsequent exposure to MTS reagents. We have previously demonstrated that a subset of mutants of translocase TM VIII (five out of seven) that displayed MTS-mediated inhibition of ATP transport could be protected from MTS modification if they were preincubated with an excess of substrate (10 times the K_m concentrations of ATP) (*19*). The mutants from TM I and II that were inhibited by all three MTS reagents were, therefore, tested for ATP protection. In these assays, single-cysteine-containing mutants were incubated with 1 mM ATP followed by exposure to MTS reagent. The results presented in Figure 5 demonstrate that S047C and T067C displayed substantial protection by 1 mM ATP. These results support the idea that the MTS reagent gains access to the mutated cysteine residue via the water-filled channel through which ATP is translocated.

The Highly Conserved "Phenylalanine Face" on TM III Is Important for Translocase Activity. There is a highly conserved clustering of four phenylalanine residues on one face of TM III (Figure 3). Of the five single-cysteine-

Mutant	TM	Near WFC	S/T Inhibition Whole Cell	A Inhibition Whole Cell
S047C	I	Yes	Yes	Yes
T067C	II	Yes	Yes	Yes
M078C	II	Yes	No	Yes
A109C	III	N/A	No	Yes

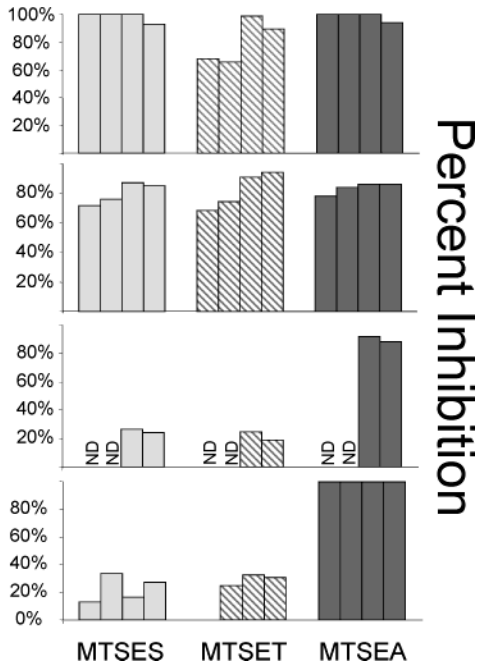

FIGURE 4: Effect of MTS reagents on ATP transport in ISO vesicles. Data are expressed as percent inhibition of control (without MTS reagents) values. Samples were assayed at 1, 2, 9, and 10 min for each of the MTS reagents with the exception of M078C where only 9 and 10 min samples were taken. Abbreviations: WFC, putative water-filled channel; S, MTSES; T, MTSET; A, MTSEA; N/A, not applicable; ND, not determined.

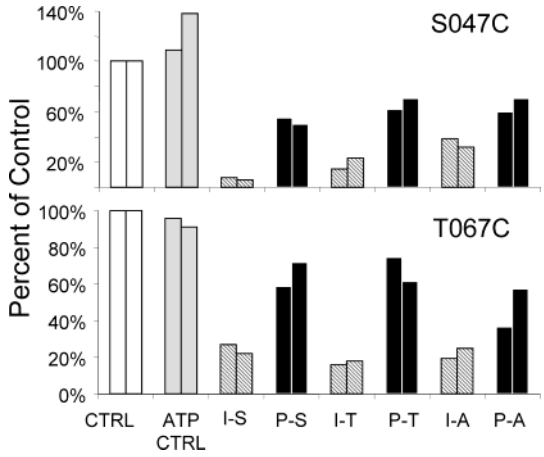

FIGURE 5: Protection from MTS reagent inhibition by ATP. Cells were exposed to the following conditions before the addition of [α-^{32}P]ATP: (i) untreated cells were preincubated in the presence (ATP CTRL, gray bars) or absence (CTRL, white bars) of 1 mM ATP (control conditions); (ii) cells were preincubated in the presence of 0.5 mM MTSES (I-S), 0.1 mM MTSET (I-T), or 0.05 mM MTSEA (I-A) (inhibited condition, hatched bars); and (iii) cells were preincubated with 1 mM ATP for 2 min followed by the addition of the above concentration of MTS reagent (P-S, P-T, P-A; protected condition, black bars). After 10 min of incubation at 34 °C, cysteine was added to a final concentration of 2 mM to inactivate residual MTS reagent. Uptake assays were then carried out at a final ATP concentration of 1 mM (1−2 μCi/mL [α-^{32}P]-ATP) with samples being removed, filtered, and washed after 9 and 10 min. Only mutants S047C and T067C that displayed protection are shown.

containing mutants in TM III generated by this study that were severely impaired for ATP transport, three were in this phenylalanine cluster (Figure 2, bottom panel). The observed detrimental effect of substitution of phenylalanine residues with cysteine may indicate that some specific property of phenylalanine is important at these positions or that an aromatic and/or hydrophobic moiety is required at these specific positions. We attempted to resolve this issue by replacing the four phenylalanine residues that constitute this essential face of TM III with an aromatic residue, tyrosine, or a hydrophobic residue, isoleucine. Three of the resulting tyrosine substitution mutants, F094Y, F101Y, and F105Y, failed to display any substantial ATP transport activity, indicating that something other than the aromatic property of phenylalanine may be required at these positions. The F108Y mutant was 13-fold more active than the corresponding F108C mutant. However, F108Y was still only 13% as active as the cysteine-less parent protein. None of the isoleucine substitution mutants show a substantial increase in ATP transport. Immunoblot analysis using anti-Tlc antisera confirmed that the lack of ATP transport activity observed in the substitution mutants was not the result of abrogated protein expression (data not shown). In toto, these results indicate that some particular property of these phenylalanine residues, not shared by tyrosine, isoleucine, or cysteine, is important to the contribution of TM III to the overall structure and function of translocase.

DISCUSSION

In the present and previous study (*19*) we demonstrated that TM I, II, and VIII are accessible to the aqueous environment and are, therefore, candidates contributing to the structure of the ATP translocation pathway. This was done by constructing a bank of single-cysteine-containing mutants of the *R. prowazekii tlc1* gene and demonstrating an inhibition of ATP uptake after exposure to charged MTS compounds. The results of the present study also demonstrate that TM III does not contain residues that are reactive with charged MTS reagents and is, therefore, unlikely to directly

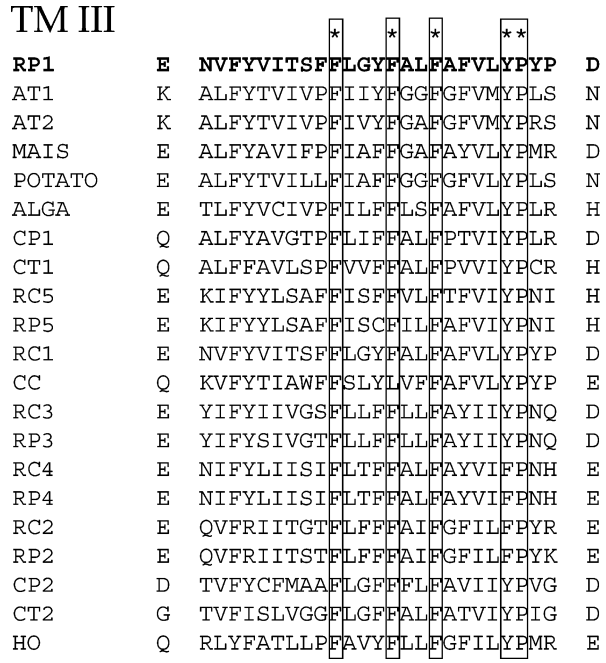

FIGURE 6: Alignment of the TM III protein sequence from rickettsial-like ATP/ADP translocases. The boundary residues are spaced away from the 25 putative residues of TM III. Mutants that were inactivated by cysteine substitution are boxed and denoted with an asterisk. Abbreviations: RP, *R. prowazekii*; AT, *A. thaliana*; MAIS, *Zea mays*; POTATO, *Solanum tuberosum*; ALGA, *Galdieria sulphuraria*; CP, *Chlamydia pneumoniae*; CT, *C. trachomatis*; RC, *R. conorii*; CC, *C. caryophilus*; and HO, *Holospora obtusa*.

contribute to the formation of an aqueous channel. Our results from whole cells were confirmed by results from ISO vesicles of seven mutants.

An alignment of ATP/ADP translocase TM III regions from various organisms including that from a plant (*Arabidopsis thaliana*), a *Chlamydia* (*Chlamydia trachomatis*), an orthologue of the rickettsial Tlc1 (*Caedibacter caryophilus*), and other rickettsial Tlc homologues shows that three of five inactive mutants in TM III identified in this study are perfectly conserved and the other two inactive mutants demonstrate a high degree of similarity (Figure 6). The fact that substitution of phenylalanine residues with cysteine, isoleucine (a hydrophobic amino acid), or tyrosine (an aromatic amino acid) resulted in a substantial reduction of ATP transport activity indicates that some specific property of phenylalanine aside from its aromatic and hydrophobic properties is critical to Tlc activity.

In addition, TM I and II each contain a single charged amino acid (R046 and K066, respectively) whose cysteine substitution resulted in an ablation of activity that is not attributable to abrogated protein expression (*7*). Having a charged residue in a transmembrane domain would be a strong indication that at least a portion of this region is exposed to the aqueous environment to house this charge. Indeed, these residues are on the same face as those residues accessible to all three MTS reagents (see Figure 3). The present study confirmed our previous results for mutants R046C and K066C in which modification with MTSET or MTSEA, which would be predicted to reintroduce a positively charged moiety to these positions, did not restore ATP transport activity (Figure 2 and ref *7*). It is possible that although a positive charge was restored to these positions, the bulky nature of the MTS adducts inhibited ATP transport.

Helical wheel models of TM I and II demonstrate that the MTS-inhibitable mutants (as well as R046 and K066) appear as a cluster of residues on one face of the helix, suggesting that these helices contribute to the structure of the water-filled ATP translocation channel (Figure 3). Two of the MTS-sensitive sites were protected from MTS-mediated inhibition of transport when they were co-incubated with 1 mM ATP (10 times the K_m) (Figure 5). It should be noted that the ATP typically present in the cytosol at about 5 mM is not sufficient to provide protection for these mutants. We postulate that the translocation pathway adjacent to these residues is empty of substrate until an exchangeable substrate is present on the other side of the channel. Moreover, we would be unable to observe protection in those cases where the residue is exposed to the cytosolic ATP. Because of the obligate exchange nature of translocase, experiments without intracellular ATP cannot be performed.

The four TM domains analyzed to date paint a picture of diversity among the TM that are exposed to the aqueous environment that may represent the ATP translocation channel. Our previous study of TM VIII revealed a periplasmic dominance of residues that were accessible to hydrophilic MTS reagents rather than having these residues clustered on a single face of the helix. In contrast, TM I and II both possess MTS-accessible residues that are clustered on a single face of each helix, with an apparent periodicity predicted for an α-helical TM domain. Although TM III does not appear to be exposed to the aqueous environment, it is clear that some property of the Phe residues (again clustered on one face of the helix) not shared by tyrosine, isoleucine, or cysteine is important to ATP transport. We hypothesize that TM I, II, and VIII, but not III, of the *R. prowazekii* ATP/ADP translocase contribute to the structure of the aqueous ATP translocation channel.

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