Cysteine-Scanning Mutagenesis and Thiol Modification of the *Rickettsia prowazekii* ATP/ADP Translocase: Characterization of TMs IV−VII and IX−XII and Their Accessibility to the Aqueous Translocation Pathway†

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ABSTRACT: We have determined the accessibility of the *Rickettsia prowazekii* ATP/ADP translocase transmembrane domains (TMs) IV−VII and IX−XII to the putative, water-filled ATP translocation pathway. A library of 177 independent mutants, each with a single cysteine substitution, was expressed in *Escherichia coli*, and those with substantial ATP transport activity were assayed for inhibition by thiol-reactive, methanethiosulfonate (MTS) reagents. The MTS reagents used were MTSES (negatively charged), MTSET (positively charged), and MTSEA (amphipathic). Inhibition of ATP transport by a charged MTS reagent indicates the exposure of a TM to the water-filled ATP translocation pathway. The eight TMs characterized in this study had 32 mutants with no assayable transport activity, indicating that cysteine substitution at these positions is not tolerated. ATP transport proficient mutants in TMs IV, V, VII, X, and XI were inhibited by charged MTS reagents, indicating that these TMs are exposed to the aqueous ATP translocation pathway, which is a pattern similar to those of TMs I, II (Alexeyev, M. F. (2004) *Biochemistry 43*, 6995−7002), and VIII (Winkler, H. H. (2003) *Biochemistry 42*, 12562−12569). Conversely, ATP-transport-proficient mutants in TMs VI, IX, and XII were not inhibited by charged MTS reagents, indicating that these TMs are sequestered from the aqueous environment, which is a pattern similar to that of TM III (Alexeyev, M. F. (2004) *Biochemistry 43*, 6995−7002). Preexposure of several MTS-sensitive mutants in TMs V, VII, X, and XI to ATP concentrations 10 times the K_m resulted in protection from MTS-mediated inhibition; thus, confirming exposure of these TMs to the aqueous ATP translocation pathway, a pattern of protection similar to that observed for TMs I, II, and VIII.

Rickettsia prowazekii is the agent of epidemic typhus and is a gram-negative, obligate intracellular α-proteobacterium that grows only within eukaryotic host-cell cytoplasm unbound by a host-derived membrane structure (*1*, *2*). As an evolutionary response to living within a rich cytosolic environment, this remarkable bacterium possesses several unusual transport systems for metabolic products available in host-cell cytoplasm. For example, rickettsiae can transport ribonucleotides, nicotinamide adenine dinucleotide, UDP-glucose, and *S*-adenosylmethionine and thus do not synthesize these metabolites (*2*−*7*). Bacteria that grow in extracellular environments generally do not transport the typically large, charged metabolites normally found in cytosol and not in the external milieu. The acquisition of such transport systems presumably released the selection pressure on many rickettsial biosynthetic pathways that have been subsequently lost through reductive evolution. The result is the present day rickettsial genome that encodes only 834 open reading frames and has a high percentage of noncoding DNA (*8*).

The ATP/ADP translocase of *R. prowazekii* (Tlc1[1]) is a membrane-bound, obligate exchange transport system that catalyzes the exchange of bacterial ADP for host-cell ATP as a source of high-energy phosphate and not the acquisition of adenylate. Tlc1 is selective for ATP and ADP and does not transport other ribonucleotides or any deoxyribonucleotides (*9*, *10*). The transport of ATP/ADP by the rickettsial Tlc1 has been characterized in both purified rickettsiae and *Escherichia coli* that express a plasmid-borne rickettsial *tlc1* gene in trans (*10*−*18*). The ATP/ADP translocase belongs to the ATP:ADP antiporter (AAA) family of transporters, a distinct group of 12 TM-containing secondary transporters as classified by Saier (*19*). Many obligate intracellular bacteria investigated to date, including the human pathogens, rickettsiae, and chlamydiae, and the protistic endosymbionts, holospora, and caedibacter, have been found to possess ATP/ADP translocases similar in function and amino acid

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[1] Abbreviations: TM, transmembrane domain; 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; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; KPi, 50 mM potassium phosphate buffer at pH 7.5; $LBAp^{100}$, Luria−Bertani media supplemented with 100 μg mL^{-1} of ampicillin; Tlc1, the *Rickettsia prowazekii* ATP/ADP translocase.

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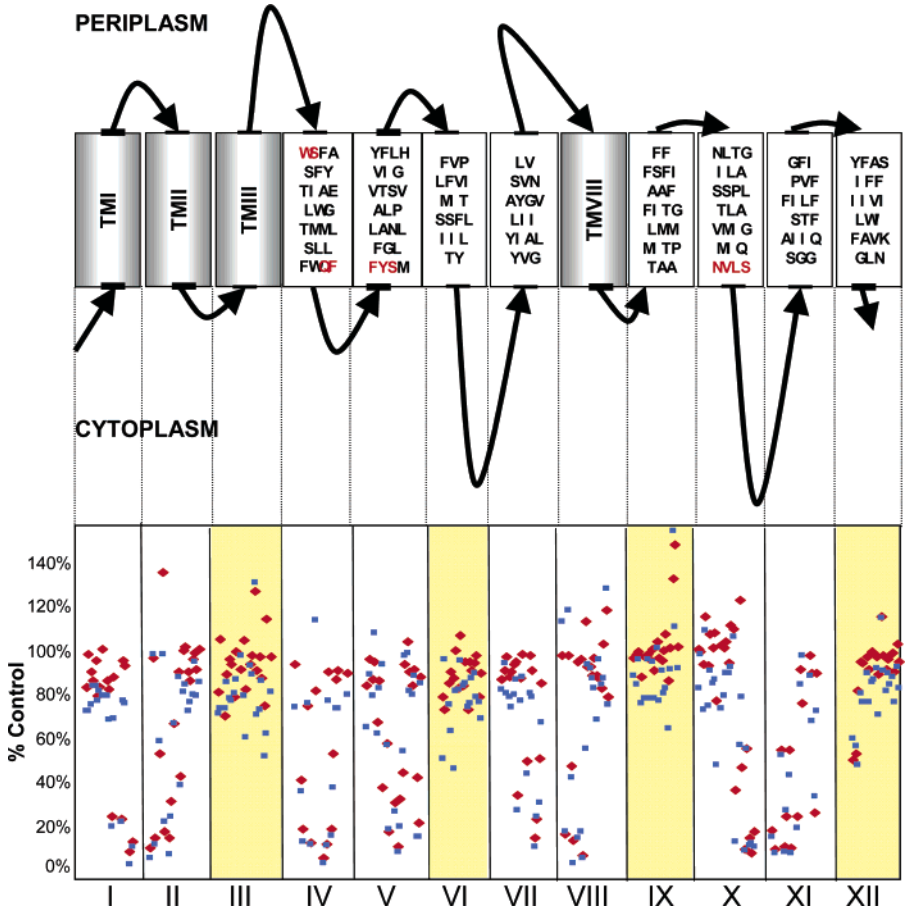

FIGURE 1: Summary of MTSES- and MTSET-mediated inhibition of the rickettsial ATP/ADP translocase single-cysteine-substitution mutants. (Top) Model of the rickettsial ATP/ADP translocase showing the residues mutated and tested in this study. The hydrophilic loops connecting the 12 transmembrane regions have lengths proportional to the number of residues in each loop. If present, charged residues are depicted on the cytoplasmic and periplasmic borders of each TM region by single-stacked (one charged residue) or double-stacked black bars (two charged residues). The arrows originate from the *N*-terminus and end at the *C*-terminus. To ensure maximal coverage, TMs IV, V, and X were extended beyond their predicted length (shown in red) as described in the text. Analyses of TMs I−III and VIII were previously reported. (Bottom) Overview of inhibition by charged MTS compounds for all TMs. Data for TMs I−III and VIII were previously reported (*27*, *28*). Inhibition by MTSES (red diamonds) and MTSET (blue squares), denoted as percent of control, is indicated for all TMs including those investigated in previous studies. TMs that exhibited limited accessibility to charged MTS reagents are shaded in yellow.

sequence (*20*−*22*). Curiously, although ATP/ADP translocases have been identified in the plastids of plants ranging from land plants to red algae (*23*, *24*), they are not found in cyanobacteria, the proposed evolutionary predecessors of plastids. Furthermore, despite the close evolutionary link between rickettsiae and mitochondria (*8*, *25*), the mitochondrial ADP/ATP transporter shares no sequence homology and little structural similarity with rickettsial ATP/ADP translocase. In fact, their functions are reversed because rickettsiae take ATP from the host cell, whereas the mitochondrion provides ATP to the host cell (*23*, *26*).

The goal of this study [and the two preceding studies (*27*, *28*)] is to examine the membrane topology of Tlc1 and identify transmembrane domains that contribute to the aqueous ATP translocation pathway. Ideally, studies of this nature employ X-ray crystallographic analyis to elucidate protein structure. However, membrane proteins often prove refractory to such analyses, save for examples of recent notable progress that has been made by solving high-resolution structures of the lactose permease (*29*), glycerol-3-phosphate transporter (*30*), and the mitochondrial ADP/ATP carrier (*31*). Our laboratory employs alternative genetic and biochemical approaches to study the Tlc1 structure. Dual *pho-lac* reporters fused to the *tlc1* gene and antibody-accessibility studies demonstrated that the ATP/ADP translocase has 12 transmembrane regions (TMs), 7 hydrophilic loops exposed to the cytoplasm, and 6 loops exposed to the periplasm (*14*, *16*) (Figure 1). This article is the final installment in a series that began with an analysis of TM VIII (*28*) and TMs I−III (*27*) that demonstrated that TMs I, II, and VIII are constituents of the water-filled ATP translocation pathway, whereas TM III is not. We now present the characterization of remaining TMs IV−VII and IX−XII and their accessibility to the aqueous pathway that must exist to transport the ATP/ADP across the membrane.

To this end, a cysteine-less mutant of the *R. prowazekii tlc1* gene that possesses 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) (*12*) was subjected to site-directed mutagenesis to generate a library of 177 single-cysteine-substitution mutants in these eight TMs. All single cysteine-containing Tlc1 mutants were assayed for ATP transport (using [α^{32}P]ATP as a tracer) (Figure 2). If a mutant was unable to transport ATP or displayed very low activity, then it was not tested for MTS-mediated inhibition. Single cysteine-containing mutants that exhibited measurable transport activity were exposed to a hydrophilic MTS reagent to

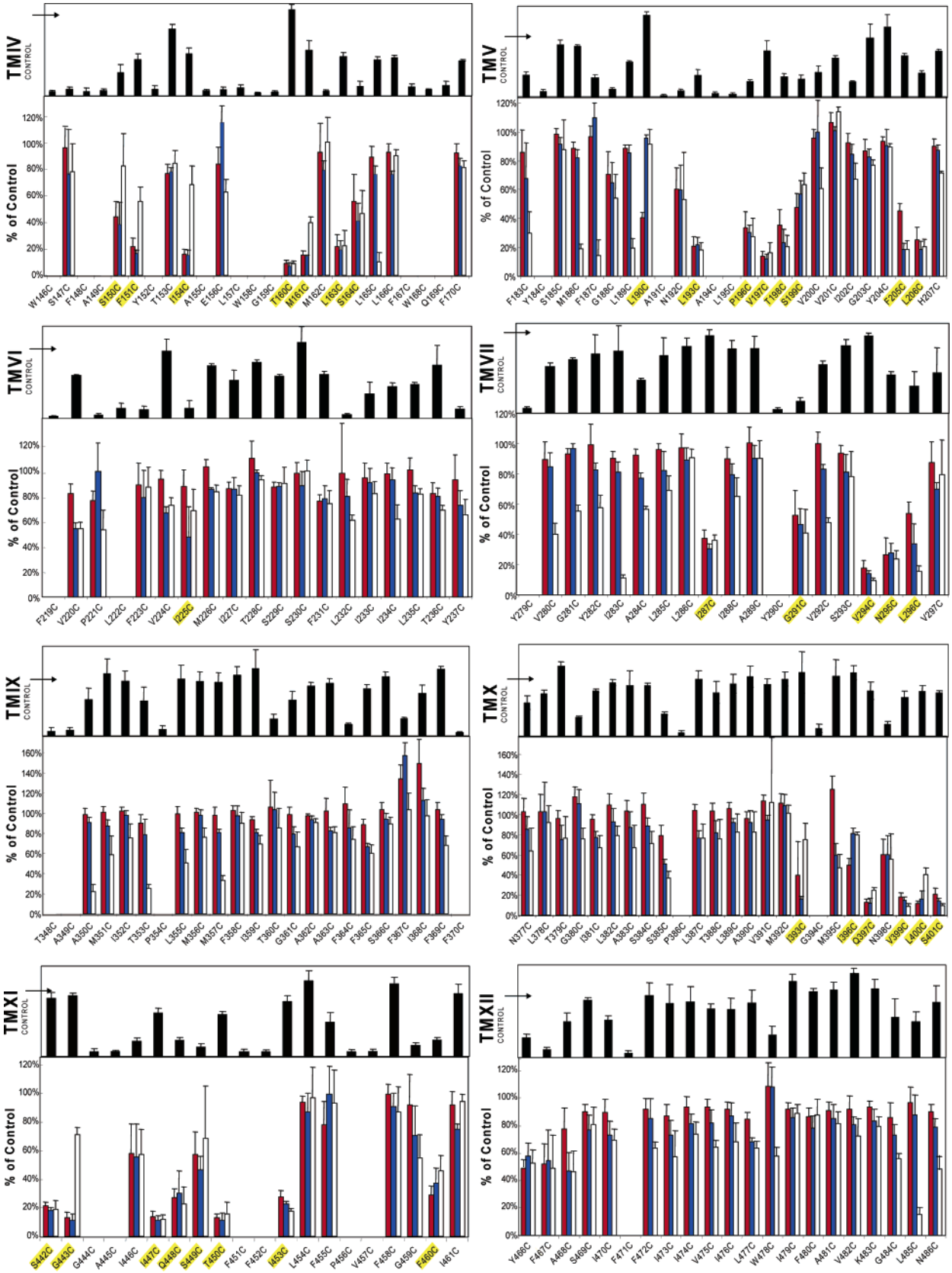

FIGURE 2: ATP transport and MTS-mediated inhibition for single-cysteine-substitution mutants within TMs IV−VII and IX−XII. The upper portion of each panel, which includes the corresponding TM label, shows the ability of single-cysteine-substituted mutants to transport ATP, which is expressed as a percent of the cysteine-less parent (about 4 nmol mg protein^{-1} min^{-1} (*12*)) with the arrow denoting 100%. 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. The lower portion of each panel shows the effect of 10 mM MTSES (red bars), MTSET (blue bars), and MTSEA (white bars) on the ATP transport activity of each single-cysteine-substituted mutant. Activity is expressed as a percent of control (no MTS reagent) with standard deviations of quadruplicate values from at least two independent experiments. Mutants highlighted in yellow demonstrated significant inhibition by charged MTS reagents.

identify TMs that contribute to the aqueous ATP translocation pathway. Sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) and [2-(trimethylammonium) ethyl] methanethiosulfonate bromide (MTSET) reagents are charged and cannot enter the lipid portion of the membrane. 2-Aminoethyl methanethiosulfonate hydrobromide (MTSEA), in contrast, can become un-ionized and potentially gain access to both aqueous and lipid domains (*32*). If ATP transport activity in several single-cysteine-containing mutants from a given TM was ablated upon exposure to either or both of the charged, hydrophilic MTS reagents, then we concluded that the TM is exposed to the ATP translocation pathway. Mutants that had the ATP transport inhibited by charged MTS compounds were also tested to determine whether the presence of a substrate (i.e., ATP) in the translocation pathway would protect these residues from the hydrophilic reagents. Similar strategies have been applied to studies examining the membrane topology of transporters in other families of transport proteins (*33*−*43*). It is important to note that regardless of the technique employed, all of these studies must assume that cysteine substitution does not grossly alter native protein conformation.

MATERIALS AND METHODS

Single-Cysteine-Substitution Mutant Construction. The construction of a bank of single-cysteine-substitution mutants corresponding to each amino acid residue in TMs IV−VII and IX−XII was undertaken as described in previous studies (*27*, *28*). Briefly, site-directed mutagenesis was performed using pMA613ΔC (referred to as the cysteine-less parent) (*13*) as a template for megaprimer PCR (*44*). In this vector, the two native cysteine codons in the Tlc1 open reading frame have been replaced with alanine, and the coding sequence of each TM is flanked by unique restriction endonuclease recognition sites. This allowed for the excision and site-directed mutagenesis of small regions of Tlc1-encoding DNA, which resulted in a low risk of secondary mutations introduced during PCR. All mutations (and the lack of undesired secondary mutations) were confirmed by sequencing. Mutagenized regions were reintroduced into pMA613ΔC, 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* λ- *thi-1 gyrA96 relA1*) and selected on $LBAp^{100}$ agar plates. Single-cysteine-containing mutant plasmids were isolated and introduced into a chemically competent C41 strain of *E. coli* (a derivative of BL21(DE3) (*45*)) to allow for IPTG-inducible expression and assay of ATP transport. The resulting library is composed of mutants in which each of the amino acid residues of TMs IV−VII and IX−XII (Figure 1) has been replaced, one-at-a-time, with cysteine. The MEMSTAT, HMMTOP, TMHMM, PredictProtein, and Phobius programs (*46*−*50*) aided in predicting the amino acids that constitute the transmembrane segments of translocase. Previous studies of Tlc1 topology by *pho-lac* dual-reporter fusion and antibody-accessibility methods (*14*, *16*) support these predictions. Where possible, the predicted TM length was extended so that it was bounded by charged amino acid residues (K, R, E, or D). To ensure maximal coverage, TMs IV, V, and X were also extended beyond their predicted length because there were no charged amino acid residues to unambiguously define a boundary. In the case of TMs IV and V, the extension mutants were either inactive or not inhibited. In the case of TM X, three of four extension mutants were inhibited by charged MTS reagents. This interesting result is discussed below. Finally, it is important to note that recent, high-resolution structural analyses of membrane transport proteins has demonstrated that TMs can be of varying lengths and do not always adhere to the convention that a TM is 19−21 residues in length (*29*, *30*). Therefore, although it seems unlikely that the TMs would extend beyond the boundaries selected in this study, it is entirely possible that they could be shorter.

Assay of Single-Cysteine-Substitution Mutants for MTS-Mediated Inhibition of ATP Transport. All of the single-cysteine-substitution mutants that displayed adequate ATP transport activity were tested for their ability to transport ATP after being exposed to MTS reagents as previously described (*28*). Mutants were tested for MTS-inhibition, if the transport activity data were repeatable (a SD <25% of the mean), even if the activity was low. Mutants that did not satisfy this criterion (e.g., mutants with low transport activity and a SD >25% of the mean) were not tested for MTS inhibition. Because of the large number of mutants being tested, the protocol was modified for high throughput using a Perkin-Elmer eight-tip HP Multiprobe II EX liquid handling system. Bacteria were routinely grown in Luria−Bertani broth, supplemented with 100 μg mL^{-1} of ampicillin ($LBAp^{100}$) at 37 °C and aeration in Lid-Bac tubes (Eppendorf Thermomixer R set at 1400 rpm). Overnight cultures of *E. coli* C41 harboring either the parent (cysteine-less translocase, pMA613ΔC) or the mutant plasmids grown in $LBAp^{100}$ were diluted into fresh $LBAp^{100}$ media, grown to an OD_{600} of 0.4, and expression-induced by the addition of 1 mM IPTG. After an hour of induction, the bacteria were washed and suspended to their original volume in KPi (all operations on ice). Bacteria were incubated with or without 10 mM MTSES, MTSET, or MTSEA for 10 min at 34 °C, followed by the addition of [α-^{32}P]ATP to initiate uptake (final concentration of 50 μM, 1 μCi mL^{-1}). ATP transport was assayed at 9 and 10 min by filtration and washed on Millipore HA filters, as previously described (*28*, *51*), using 96-well filter plates. The radioactivity on each filter was measured by Perkin-Elmer Cyclone phosphor image analysis to determine the level of ATP uptake. Sensitivity to MTS reagents was expressed by comparison of the transport ability of each single-cysteine-containing mutant under control (no MTS) and MTS-treated conditions. MTSES, MTSET, and MTSEA compounds were purchased from Toronto Research Chemical Inc. (North York, Ontario, Canada), and stock solutions dissolved in KPi were prepared immediately prior to use because of their instability in aqueous solution.

When inhibition data from all 222 assayable mutants in all 12 TMs is viewed collectively (Figure 1), there seems to be a clustering of data points occurring between 70 and 110% of control; this clustering likely represents variation in the control values. Nonspecific inhibition of the C41 expression strain harboring the cysteine-less parent plasmid was about 10% when 10 mM concentrations of MTS reagents were present. Furthermore, the plasmid-less C41 expression strain displayed about 8% nonspecific uptake of the radiolabel when incubated with [α^{32}P]ATP. Therefore, we conservatively demanded that a mutant must exhibit at least 50% inhibition to be categorized as inhibited. Also, there were

mutants in every TM that displayed moderate levels of inhibition (51−70% of non-MTS control) that were not discriminatory as to the accessibility of the TM to the water-filled translocation pathway. Previous studies of the mitochondrial citrate transport protein by Kaplan's group (*52*, *53*) indicate that the determination of pseudo first-order rate constants of inhibition by MTS reagents is required for the full interpretation of this type of moderate inhibition data. Considering the goal of the present study to determine TMs that contribute to the water-filled ATP translocation pathway, data on mutants with moderate inhibition are presented in Figure 2 but not discussed further. Results reported are the averages of two to six separate experiments with quadruplicate points in each experiment.

ATP Protection Assays. Our previous studies of translocase TMs I−III and VIII demonstrated that the presence of excess substrate (i.e., 1 mM ATP) protected some mutants from MTS-mediated transport inhibition (*27*, *28*) and served as confirmation that a given TM is exposed to the aqueous environment. Therefore, mutants that displayed an inhibition of ATP transport when exposed to high concentrations of MTS reagents (10 mM MTSES and MTSET) were tested for protection by 1 mM ATP in the presence of low concentrations of MTS reagents (0.5 mM MTSES or 0.1 mM MTSET). In several cases, low concentrations of MTS reagents were not sufficient to inhibit ATP uptake. Cells were grown, induced, and washed as above and exposed to the following preincubation conditions before the addition of [α-^{32}P]ATP: (i) preincubation without MTS compounds or ATP (control conditions); (ii) preincubation with 0.5 mM MTSES or 0.1 mM MTSET (inhibited conditions); and (iii) preincubation with 1 mM ATP for 1 min, followed by the addition of the above concentrations of MTS reagents (protected conditions). After 10 min at 34 °C, uptake assays were carried out at a final ATP concentration of 1 mM (1 μCi mL^{-1} [α-^{32}P]ATP) as above.

RESULTS AND DISCUSSION

Transport-Negative Mutants. In the Tlc1 TMs characterized in this and previous studies (*27*, *28*) there were residues in which the single-cysteine substitution caused an extensive loss of transport activity. Combining the data from all three of our studies, 44 of the 266 (17%) single-cysteine-substitution mutants tested had ATP transport activity that was too low to be measured. In the current study, 32 of the 177 (18%) single-cysteine-substitution mutants tested had ATP transport activity that was too low to be measured. They are W146C, F148C, A149C, Y152C, A155C, L157C, W158C, G159C, F167C, W168C, and Q169C (in TM IV); Y184C, A191C, A194C, L195C (in TM V); F219C and L222C (in TM VI); Y279C and Y290C (in TM VII); T348C, A349C, P354C, and F370C (in TM IX); P386C and G394C (in TM X); G444C, A445C, F451C, F452C, P456C, and V457C (in TM XI); and F471C (in TM XII). TM IV possessed the most nonassayable mutants (11 in total), a 3-fold increase over the average number of inactive mutants for all TMs. An analysis of the average activity (% of control) for all mutants in a given TM demonstrated that TM IV (as expected from the large number of nonassayable mutants) was most adversely affected by cysteine-substitution (an average of 32% of control). TMs V, VI, and XI were moderately affected (averages of 56, 61, and 53% of control, respectively). The remainder of the TMs assayed in this study were affected to a much lesser extent (averages greater than 80% of control). The observed reduction of activity could indicate a direct ablation of transport activity, reduced expression, decreased stability, or decreased insertion. A discussion of the mechanism of inactivation of transport-negative mutants is not germane here because this study is focused on identifying TMs that define a part of a water-filled ATP translocation pathway by assaying the effect of charged MTS reagents on ATP transport. The remaining mutants (222 in all studies, 145 in the present study) displayed an adequate level of ATP transport that could be reliably measured and characterized with respect to MTS-mediated inhibition of transport activity (Figure 2).

Overview. Considering the large amount of data generated by these studies, the results from all 12 TMs are summarized and presented collectively in Figure 1 to give the reader a general overview. The data in Figure 1 shows the mean inhibition caused by the addition of the two charged MTS reagents (MTSES and MTSET but not the amphipathic MTSEA). In response to MTSES/MTSET exposure, a striking pattern of almost total inhibition is exhibited by mutants in TMs I, II, IV, V, VII, VIII, X, and XI suggesting that portions of these TMs are exposed to an aqueous environment. In contrast, TMs III, VI, IX, and XII display a different pattern. These TMs are suggested to be entirely embedded in the lipid matrix or have their amino acid side chains closely associated with the side chains of a neighboring TM and are therefore sequestered from the aqueous environment and charged MTS reagents (Figure 1). The following is a detailed analysis for each of the single-cysteine-substitution mutants characterized in this study.

Analysis of Each TM. In TM IV, 25 mutants were tested, and 5 basic patterns were evident (Figure 2). (*i*) ATP transport was inhibited by all three MTS reagents in mutants T160C, M161C, and L163C, providing evidence that these residues are exposed to an aqueous ATP translocation pathway. (*ii*) Mutants S150C, F151C, and I154C were inhibited by MTSES and MTSET but not MTSEA, again suggesting a role for TM IV in formation of the ATP translocation pathway. It is possible that the smaller sized MTSEA was unable to sterically hinder the transport of ATP. (*iii*) Mutant S164C was inhibited by MTSET and MTSEA but exhibited only low levels of inhibition by MTSES, which indicates a greater sensitivity of S164C to the presence of positively charged adducts and an association of TM IV with an aqueous pathway. (*iv*) MTS-mediated inhibition of mutant L165C was only observed in the presence of MTSEA, demonstrating that either MTSES and MTSET adducts are not inhibitory or charged compounds do not have access to this position. (*v*) ATP transport was not affected by exposure to any of the MTS reagents in mutants S147C, T153C, M162C, L166C, and F170C suggesting that formation of cysteine-MTS adducts at these positions does not adversely affect ATP transport or that these positions are not accessible to MTS reagents. The data from the remainder of the mutants were not discriminatory in that these mutants either had no ATP transport activity or were not inhibited more than 50% by the MTS reagents. On the basis of these data showing that seven mutants displayed greater than 50% inhibition by charged MTS reagents, we conclude that TM IV is exposed to the water-filled ATP translocation pathway.

In TM V, 25 mutants were tested, and 4 patterns of inhibition were observed (Figure 2). (*i*) Inhibition of ATP transport by all three MTS compounds was observed in mutants L193C, P196C, V197C, T198C, F205C, and L206C, indicating that these residues were exposed to an aqueous environment. (*ii*) Mutants L190C and S199C were inhibited only by MTSES, suggesting an inhibitory effect of negatively charged adducts. (*iii*) Mutants F183C, M186C, F187C, and L189C were inhibited only by MTSEA, indicating either that MTSES and MTSET adducts were not inhibitory or that these mutants were inaccessible to charged MTS compounds. (*iv*) Mutants S185C, V201C, G203C, Y204C, and H207C were not inhibited by any MTS compound, indicating either that MTS adducts are not inhibitory or that the compounds do not have access to these positions. The data from the remainder of the mutants was not discriminatory for reasons described above. On the basis of these data showing that eight mutants displayed greater than 50% inhibition by charged MTS reagents, we conclude that TM V is exposed to the water-filled ATP translocation pathway.

In TM VI, 19 mutants were tested. TM VI displayed a pattern similar to those of TM III, IX, and XII (Figure 1 (*27*), and see below) in that not one of the mutants tested was inhibited by all three MTS compounds (Figure 2). Mutants F223C, M226C, I227C, T228C, S229C, S230C, F231C, I233C, and L235C were not inhibited by any of the MTS compounds. Mutant I225C was modestly inhibited only by MTSET. It is likely that a detailed resolution of TM helix packing is required to understand fully the modest level of inhibition of a single mutant by a charged MTS reagent in a TM that otherwise appears to be sequestered from the aqueous translocation pore. Perhaps a close association of this region of TM VI with an adjacent, water-facing TM could account for the modest but unexpected reactivity of this mutant. The data from the remainder of the mutants was not discriminatory. On the basis of the similarity of the inhibition patterns of TMs III, VI, IX, and XII (Figure 1 (*27*), and see below), these results indicate that TM VI is not exposed to the water-filled ATP translocation pathway. However, considering that none of the mutants in TM VI are inhibitable by MTSEA, there is the possibility that charged MTS reagents are reactive but these adducts are not inhibitory. This alternative is always a potential limitation of structural analyses that use inhibition of transport activity as an indicator of reactivity with charged MTS reagents. Compare this to methodologies that assay charged MTS reagents for the ability to protect single-cysteine-substitution mutants of a transport protein from reacting with radiolabeled *N*-ethylmaleimide to elucidate structure (*43*, *54*−*56*). Although both techniques have advantages and disadvantages, the high-throughput assay of the transport activity of a large number of mutants allows a relatively rapid survey for water-exposed TMs. Assays that determine thiol reactivity independent of transport activity are limited in those instances where single cysteine mutants are completely unreactive with *N*-ethylmaleimide (*43*).

In TM VII, 19 mutants were tested, and 4 basic patterns of inhibition were observed (Figure 2). (*i*) ATP transport was inhibited by all three MTS compounds in mutants I287C, V294C, and N295C, providing strong evidence that these residues are exposed to the water-filled ATP translocation pathway. (*ii*) Mutants G291C and L296C were inhibited by MTSET and MTSEA, indicating a greater sensitivity to positively charged adducts. (*iii*) Mutants V280C, I283C, and V292C were inhibited only by MTSEA, thus indicating either that charged MTS reagents do not have access to these residues or that their adducts are not inhibitory. (*iv*) MTS reagents did not inhibit ATP transport in mutants L286C, A289C, and S293C. The data from the remainder of the mutants was not discriminatory. On the basis of these data showing that five mutants displayed greater than 50% inhibition by charged MTS reagents, we conclude that TM VII is exposed to the water-filled ATP translocation pathway.

In TM IX, 23 mutants were tested. The inhibition pattern observed for TM IX closely resembles those of TMs III, VI, and XII (Figure 1 (*27*), and see below) in which no mutant was inhibited by all three MTS compounds (Figure 2). Three mutants were inhibited only by MTSEA (A350C, T353C, and M357C). Ten mutants were not inhibited by any of the MTS reagents (I352C, M356C, F358C, T360C, A362C, A363C, F364C, S366C, F367C, and I368C). The data from the remainder of the mutants was not discriminatory. As in TMs III, VI, and XII, these results indicate that TM IX is not exposed to the water-filled translocation pathway.

In TM X, 25 mutants were tested, and 5 basic inhibition profiles were evident (Figure 2**)**. (*i*) Four mutants (Q397C, V399C, L400C, and S401C) were inhibited by all three MTS compounds, indicating the presence of an aqueous access route to these residues. (*ii*) Mutant I393C was inhibited by both MTSES and MTSET but not the smaller MTSEA, indicating access to TM X via an aqueous pathway and susceptibility to the larger, charged adducts. (*iii*) Mutant I396C was inhibited when exposed to MTSES but not MTSET or MTSEA, indicating access to the aqueous pathway and sensitivity to the negatively charged adducts. (*iv*) Two mutants, S385C and M395C, were inhibited by MTSEA but not by MTSES or MTSET, demonstrating either that MTSES and MTSET adducts are not inhibitory or that charged compounds do not have access to these positions. (*v*) Eleven mutants were unaffected by any MTS compounds (L378C, T379C, G380C, L382C, S384C, L387C, T388C, L389C, A390C, V391C, and M392C). The data from the remainder of the mutants was not discriminatory. The identification of six mutants (I393C, I396C, Q397C, V399C, L400C, and S401C) that are inhibitable by charged MTS reagents suggests that TM X forms a part of a water-filled pathway. However, although three of these six inhibitable mutants (I393C, I396C, and Q397C) are predicted by an in silico analysis to be in a TM domain, the other three are predicted to reside in the adjacent cytoplasmic loop (Figure 1). To have a putative TM X that terminates unambiguously on the cytoplasmic side at a charged residue (K402), the additional mutants (N398C, V399C, L400C, and S401C) were constructed, and all but N398C were found to be sensitive to all three MTS compounds. Even if V399C, L400C, and S401C are in the cytoplasmic loop, the inhibition of these three mutants by charged MTS reagents suggests that TM X contributes to a water-filled translocation pathway. The logic is that an aqueous route would be required to efficiently convey the charged MTS compounds to the terminus of this transmembrane pathway. This would allow reactions with nearby aqueous-pathway-proximal mutants V399C, L400C, and S401C before the MTS compounds are

rendered inactive by dilution and reaction with cytosolic thiols.

In TM XI, 20 mutants were tested and exhibited 5 basic patterns of inhibition (Figure 2). (*i*) There were six mutants whose ability to transport ATP was inhibited by all three MTS compounds (S442C, I447C, Q448C, T450C, I453C, and F460C). (*ii*) Mutant G443C was inhibited by MTSES and MTSET but not by the smaller MTSEA. (*iv*) Mutant S449C was only inhibited by the large, positively charged MTSET adduct. (*v*) There were four mutants that were not inhibited by any of the MTS compounds (L454C, F455C, F458C, and I461C). The data from the remainder of the mutants was not discriminatory. On the basis of these data showing that eight mutants displayed greater than 50% inhibition by charged MTS reagents, we conclude that TM XI is exposed to the water-filled ATP translocation pathway.

In TM XII, 21 mutants were tested. TM XII is similar to TMs III, VI, and IX (Figure 1 (*27*), and see below) in that no mutants within this TM were inhibited by all three MTS compounds (Figure 2). ATP transport in mutant L485C was only inhibited by the amphipathic MTSEA reagent and was unaffected by MTSES or MTSET. There were nine mutants not inhibited by any of the MTS compounds (S469C, I470C, I474C, I476C, I479C, F480C, A481C, V482C, and K483C). The data from the remainder of the mutants was not discriminatory. As with TMs III, VI, and IX, the lack of substantial ATP transport inhibition by charged MTS compounds indicates that TM XII is not exposed to the aqueous ATP translocation pathway.

Inside-Out Vesicles. In their substituted-cysteine accessibility mutagenesis analysis of the mitochondrial citrate transport protein in proteoliposomes, Kaplan and co-workers used pseudo-first-order rate constants to demonstrate a gradient of MTS-mediated inhibition of citrate transport (*52*, *53*). Mutants proximal to the side from which the MTS reagent was added were more sensitive than mutants proximal to the opposite side of the liposome. In our previous studies of TMs I−III and VIII (*27*, *28*), we included the construction and assay of inside-out (ISO) vesicles to confirm and possibly extend the inhibition data and determine if a similar gradient of MTS accessibility existed. However, in every case, testing of ISO vesicles merely confirmed the results generated from whole-cell assays in that MTS-mediated inhibition of a given mutant occurred independent of the exposure to the reagent occurring from the periplasmic side (i.e., in whole cells) or the cytoplasmic side (i.e., in ISO vesicles). Our inability to observe a gradient of sensitivity similar to that demonstrated by Kaplan is most likely due to their use of pseudo first-order rate constants of inhibition. Alternatively, the differences could be inherent to their use of proteoliposomes versus whole cells or to unknown intrinsic differences in the two transporters. Regardless, experiments on ISO vesicles were not included in this study because they had not provided any new or exclusive information.

Preincubation with 1 mM ATP Is Protective against MTS-Mediated Inhibition. We have previously demonstrated that a subset of mutants of translocase TMs I, II, and VIII that displayed MTS-mediated inhibition of ATP transport could be protected from this inhibition if they were preincubated with an excess of ATP (10 times K_m concentration of the cysteine-less and wild-type translocase proteins) (*27*, *28*).

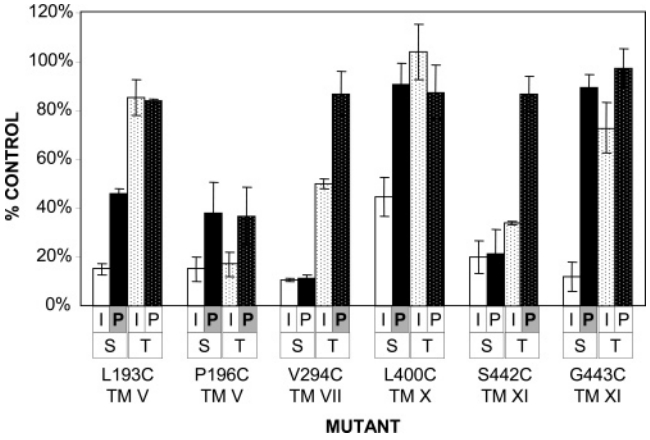

FIGURE 3: Protection from MTS reagent inhibition by ATP. Mutants (from Figure 2) that displayed inhibition of ATP transport when exposed to high concentrations of MTS reagents (10 mM MTSES and MTSET) were tested for protection by 1 mM ATP in the presence of low concentrations of MTS reagents (0.5 mM MTSES or 0.1 mM MTSET). All data are shown, even though in some cases, the low concentration of MTS reagent was not sufficient to inhibit ATP uptake under control conditions. The cells were exposed to the following conditions before the addition of [α^{32}P]ATP: (i) preincubation without MTS compounds or ATP as the control conditions; (ii) preincubation with 0.5 mM MTSES (white bars; denoted as S) or 0.1 mM MTSET (dotted bars; denoted as T) as the inhibited conditions (denoted as I); and (iii) preincubation with 1 mM ATP for 1 min, followed by the addition of the above concentrations of MTS reagents as the protected conditions (black bars; denoted as P). All transport assays were carried out at a final ATP concentration of 1 mM (1 μCi [α^{32}P]ATP). Mutants that demonstrated substantial protection are demarcated with a shaded box with the letter **P**.

The presence of excess ATP in the translocation pathway is thought to prevent the MTS compounds from having access to the mutant-cysteine residues that line this pathway. This observed protection is remarkable, considering that a transported substrate has a passing association with residues in the translocation pathway, whereas the MTS reagent forms a covalent bond. Therefore, with time, the irreversible nature of the MTS reaction will diminish the protective effects of ATP and likely contribute to the fact that only certain positions in the water-filled translocation pathway can be protected. Indeed, we had to use a low concentration of MTS reagent to observe protection.

Mutants in the present study that were inhibitable by low concentrations of MTSES and MTSET were tested for ATP protection (Figure 3). None of the MTS-inhibited mutants in TM IV were protected by 1 mM ATP. In TM V, mutant L193C demonstrated protection from MTSES-mediated inhibition, and mutant P196C demonstrated protection from both MTSES- and MTSET-mediated inhibition. In TM VII, mutant V294C exhibited protection from MTSET-mediated inhibition. In TM X, the protection of mutant L400C resulted in the restoration of activity to near control levels in the presence of MTSES, indicating that the extension of TM X to include N398, V399, L400, and S401 (discussed above) may well be justified. In TM XI, S442C was protected against MTSET-mediated inhibition, and G443C was protected against MTSES-mediated inhibition. These results support our conclusions that TMs V, VII, X, and XI are exposed to the water-filled ATP translocation pathway. A caveat of the protection assay is that cysteine-substitution

could decrease the affinity for ATP and result in the misclassification of unprotected sites.

The protected mutants illuminate an interesting point regarding the obligate exchange antiporter mechanism of Tlc1. It is curious that the concentration of ATP typically present in the cytosol (about 5 mM) is obviously not sufficient to provide protection for these mutants because inhibition is observed, whereas protection of several mutants is achieved with the addition of 1 mM ATP. (We have previously demonstrated that Tlc1 is able to catalyze an ATP/ATP exchange (*10*).) This supports the idea that the translocation pathway adjacent to these protectable residues remains devoid of substrate and thus is unprotected, until an exchangeable counter substrate is presented on the other side of the translocation pathway to initiate an exchange.

Analysis of Charged Amino Acid Residues. The presence of a charged amino acid residue within a predicted TM domain should be a strong predictor of exposure to the aqueous environment and perhaps indicate a role in coordination of the substrate in the translocation pathway. Tlc1 has four TMs that contain a single charged residue. Three of these four TMs (I, II, and IV) contain a single charged amino acid (R046, K066, and E156, respectively) whose cysteine-substitution resulted in very low or total ablation of activity with no change in protein levels in the membrane compared to that of the cysteine-less parent control (see (*15*) for R046 and K066; data not shown for E156). This lack of activity is consistent with the idea that these charged residues are critical to Tlc1 function. Intriguingly, TM XII has a positively charged residue, K483, yet our data strongly indicate that TM XII is not markedly exposed to the aqueous environment. This raises an interesting issue of the structural effect of housing a charged residue in the lipid phase. Our data show that replacement of K483 with cysteine did not have any effect on ATP transport activity in contrast to R046, K066, and E156, where replacement with cysteine abolished activity. Therefore, the positive charge in TM XII is not associated with the aqueous ATP translocation pathway and is not essential to Tlc1 structure and function. Alternatively, this is an extremely short TM domain consisting of 17 residues, and K483 resides in a loop.

Summary and Final Thoughts. We have constructed a bank of single-cysteine-containing mutants of the *R. prowazekii tlc1* gene and assayed these mutants for the inhibition of ATP uptake, after the exposure to MTS compounds, to determine transmembrane domains that are exposed to the water-filled ATP translocation pathway. The idea that the substantial transport activity of a single-cysteine-substitution mutant is indicative of near-native conformation is a central tenet of this study. Figure 1 presents an overview, combining the data generated in the present and previous studies (*27*, *28*). We have demonstrated that TMs I, II, IV, V, VII, VIII, X, and XI are accessible to the aqueous environment and are therefore candidates to form the structural components of the ATP translocation pathway (Figure 1). Conversely, TMs III, VI, IX, and XII do not contain mutants that are inhibitable by charged MTS reagents and are, therefore, unlikely to directly contribute to the formation of an aqueous translocation pathway. This conclusion is supported by the protection data presented in Figure 3.

Intriguingly, although the rickettsial Tlc1 is classified in the AAA family and not the MFS family, we have noted a striking similarity in the TMs that constitute the water-filled, substrate translocation pathway. Similar to both MFS family members, LacY and GlpT, (*29*, *30*), the rickettsial Tlc1 TMs I, II, IV, V, VII, VIII, X, and XI are exposed to the water-filled ATP translocation pathway, and TMs III, VI, IX, and XII are likely not. Future helix packing studies may strengthen the relationship between these two families of 12 TM-containing carriers by suggesting a conserved arrangement of TM domains. If so, it would be tempting to speculate that, even though the MFS and AAA families do not appear related by amino acid sequence similarity, there may be higher-order structural homology indicative of a common evolutionary ancestor.

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