Transmembrane Domain 5 of the LdNT1.1 Nucleoside Transporter Is an Amphipathic Helix That Forms Part of the Nucleoside Translocation Pathway†

Raquel Valdés, Gayatri Vasudevan, David Conklin, and Scott M. Landfear*

Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, Oregon 97239

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ABSTRACT: Transporters of the equilibrative nucleoside transporter (ENT) family promote the uptake of nucleosides, nucleobases, and a variety of therapeutic drugs in eukaryotes from protozoa to mammals. Despite its importance, the translocation pathway that mediates the internalization of these substrates has not been identified yet in any of the ENT carriers. Previous genetic studies on the LdNT1.1 nucleoside transporter from *Leishmania donovani* defined two amino acid residues in predicted transmembrane domains (TMD) 5 and 7 that may line this translocation pathway. The role of TMD5 in forming a portion of the aqueous channel was investigated using the substituted-cysteine accessibility method. A series of 22 cysteine substitution mutants spanning predicted TMD5 were created from a fully functional, cysteine-less, parental LdNT1.1. Cysteine replacement at six positions ($M_{176}C$, $T_{186}C$, $S_{187}C$, $Q_{190}C$, $V_{193}C$, and $K_{194}C$) produced permeases that were inhibited by incubation with sulfhydryl-specific methanethiosulfonate reagents, denoting their solvent accessibility to the translocation pathway. Adenosine was able to block this thiol modification, implying that access to the domain becomes restricted as a consequence of the substrate binding. Strikingly, the $Q_{190}C$ substitution interacted differentially with the substrates adenosine and uridine, suggesting that binding of adenosine but not uridine might directly occlude this position. When superimposed on a helical model, all six mutants clustered along one face of the amphipathic α-helix predicted for TMD5, strongly suggesting its involvement in the translocation pathway through LdNT1.1.

Leishmania donovani is the causative agent of visceral leishmaniasis, a devastating and often fatal disease if untreated. The parasite exhibits a digenetic lifecycle in which the extracellular, flagellated, and motile promastigote resides within the phlebotomine sandfly vector, and the intracellular, aflagellar, and nonmotile amastigote exists within the phagolysosome of macrophages and other reticuloendothelial cells of the mammalian host. Purine salvage pathways are critical to the survival of parasitic protozoa, because all these organisms lack the ability to synthesize the purine ring de novo (*1*). This metabolic disparity between parasites and their mammalian hosts, which do synthesize purines, has been exploited for rational development of improved therapies and for design of selective antiparasitic drugs (*2*). Since the initial step in purine salvage involves the translocation of preformed host purines across the parasite surface membranes, parasite nucleoside and nucleobase transporters may constitute potential targets for chemotherapy or routes for delivery of purine analogue drugs.

The LdNT1 transporters of *L. donovani* mediate the uptake of adenosine, pyrimidine nucleosides, and the cytotoxic adenosine analogue tubercidin. The *LdNT1* genetic locus encompasses two closely related genes, *LdNT1.1* and *LdNT1.2*, which were cloned by functional complementation (*3*) of an adenosine/pyrimidine transport-deficient mutant cell line, TUBA 5 (*4*). Predicted amino acid sequences and membrane topologies of LdNT1.1 and LdNT1.2 revealed that these transporter proteins are members of the equilibrative nucleoside transporter (ENT)[1] family (*5*) and exhibit significant sequence identity to the mammalian ENTs as well as the same predicted topology model encompassing 11 transmembrane domains (TMDs) (*6*) (Figure 1A). Although originally identified in mammals (*7*), the ENT permeases are now known to be widely distributed among higher and lower eukaryotes (*5*) and constitute major foci of study due to their central roles in the pharmacology of cancer, cardiovascular disease, and parasitic infections (*8*). Despite the importance of nucleoside and nucleobase transporters in cellular nutrition or as ideal conduits for the delivery of new drugs, little is known about the amino acid residues or domains that are involved in binding and/or membrane translocation of nucleosides and nucleobases.

Mutational dissection of *LdNT1.1* provided significant albeit indirect evidence that TMD5 might form part of the

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* To whom correspondence should be addressed: Phone: (503) 494-2426. Fax: (503) 494-6862. E-mail: landfear@ohsu.edu.

[1] Abbreviations: ENT, equilibrative nucleoside transporter; h, human; r, rat; LdNT, *Leishmania donovani* nucleoside transporter; TM, transmembrane; TMD, transmembrane domain; SCAM, substituted-cysteine accessibility method; MTS, methanethiosulfonate; MTSEA, MTS-ethylammonium; MTSET, MTS-ethyltrimethylammonium; MTSES, MTS-ethyl sulfonate; cys-less, cysteine-less; cys mutant, cysteine mutant; PBS, phosphate-buffered saline; ORF, open reading frame; PCR, polymerase chain reaction; SD, standard deviation; for brevity, the one-letter system for amino acids has been used: $M_{176}C$, for example, refers to the $Met_{176} \rightarrow Cys$ site-directed mutant.

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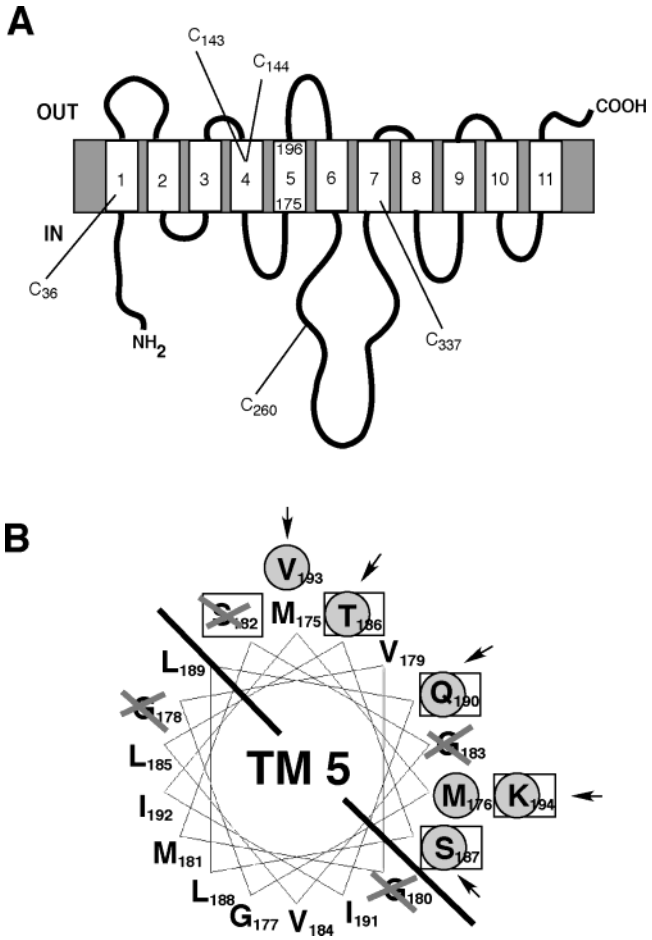

FIGURE 1: (A) Membrane topology of LdNT1.1 and location of the endogenous cysteine residues. Predicted topological model of LdNT1.1 (*3*) corresponds to that of other equilibrative nucleoside transporters (ENTs) (*5*) and encompasses 11 transmembrane domains (white rectangles), with cytoplasmic N-terminal and externally orientated C-terminal hydrophilic domains. Also shown are the positions of the five endogenous cysteine residues in the LdNT1.1 primary sequence. Numbers at the bottom and top of helix 5 indicate the amino acids predicted to initiate and terminate TMD5. (B) Helical wheel representation of helix 5. Residues modified by any of the MTS reagents are represented within filled circles and residues with hydrophilic side chains are shown within rectangles; the solid line through the helix separates the hydrophobic face from the hydrophilic face. Arrows indicate those positions that were significantly protected by adenosine, and crosses indicate positions that result in transport-incapacitating mutations when changed to cysteine. Amino acids are represented by the single letter code. The helical wheel was generated using the Wisconsin Package Version 10.3-UNIX.

translocation pathway (*9*). The examination of the molecular nature of the mutations leading to transport deficiency in the TUBA5 cell line revealed that this mutant was a compound heterozygote at the *LdNT1.1* locus containing two mutant alleles that encompassed distinct point mutations, each of which impaired transport function. One of the mutant *LdNT1.1* alleles encoded a $G_{183}D$ substitution in the hydrophilic face of the predicted TMD5. This mutant permease trafficked correctly to the cell surface, but the severe impairment in transport resulted from drastically reduced V_{max} values, apparently derived from a defect in substrate translocation and consistent with a possible blockage of the pore by the mutated residues. Moreover, a mutant transporter with a more conservative $G_{183}A$ substitution resulted in altered substrate specificity, exhibiting robust adenosine transport but almost undetectable uridine uptake, a strong suggestion that the region around G_{183} is likely to interact intimately with the substrate (*9*).

The substituted-cysteine accessibility method (SCAM) (*10*) has revolutionized the structural analysis of membrane-spanning ion channels, receptors, and transporters, being currently the method of choice for examining substrate and ligand binding sites in such polytopic membrane proteins. Reinserting single cysteine residues into functional "cysteine-less" proteins and treating the resulting "cysteine mutants" with thiol-reactive agents typified by the methanethiosulfonates (MTS) (*11*, *12*) makes it possible to elucidate membrane topologies (*13*−*16*) and to identify amino acids that are accessible to the aqueous phase (*17*−*20*). Variations of this approach have been elegantly applied to characterize the structures and ligand binding sites of an assortment of membrane proteins such as the *Escherichia coli* lactose permease (*21*) and glucose-6-phosphate transporter (UhpT) (*22*), the yeast mitochondrial citrate carrier (*23*), the human glucose transporter Glut1 (*24*−*27*), the human dopamine transporter (*28*), as well as ion channels (*29*), including the cystic fibrosis transmembrane conductance regulator (*30*) and a Ca^{2+} channel (*31*).

In the present study, we used cysteine-scanning mutagenesis in conjunction with three thiol-reactive MTS derivatives to directly address the role of TMD5 in mediating substrate translocation. These investigations constitute the first direct evidence, among members of the ENT family, for TMD5 being an amphipathic α-helix with a water-accessible face that lines the nucleoside translocation pathway. As such, these results contribute to our emerging knowledge of the structure and function of this important family of permeases.

EXPERIMENTAL PROCEDURES

Chemicals, Materials, and Reagents. Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs, Inc. (Beverly, MA), Roche Pharmaceuticals (Nutley, NJ), or Life Technologies, Inc. (Gaithersburg, MD). MTSEA, MTSET, and MTSES were purchased from Toronto Research Chemicals (Toronto, ON, Canada) or Biotium, Inc. (Hayward, CA). Radiolabeled [2,8-^{3}H]-adenosine (40 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [5,6-^{3}H]-uridine (32.3 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Hygromycin B was purchased from Roche Pharmaceuticals (Nutley, NJ) and G418 from Invitrogen Life Technologies (Carlsbad, CA). Synthetic oligonucleotides were purchased from Invitrogen. All other chemicals, materials, and reagents were of the highest commercial quality available.

Parasite Cell Culture. *L. donovani* wild-type (DI700) strain was propagated at 26 °C in RPMI medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum, 15 μg mL^{-1} hemin and 100 μM xanthine as a purine source. Null mutant Δ*ldnt1* was cultured continuously in 50 μg mL^{-1} hygromycin (Roche Pharmaceuticals, Nutley, NJ) and 1 μM tubercidin (Sigma Chemical Co., St Louis, MO). Cell lines generated by transfection of Δ*ldnt1* with pX63NeoRI constructs (described below) were selected and maintained in 100 μg mL^{-1} G418 (Invitrogen Life Technologies, Carlsbad, CA) and 50 μg mL^{-1} hygromycin.

Site-Directed Mutagenesis and Plasmid Constructs. The cysteine-less (cys-less) transporter and individual cysteine mutants (cys mutants) within TMD5 of the LdNT1.1 permease (codons 175−196) were generated by the QuickChange site-directed mutagenesis protocol, a polymerase chain reaction-based mutagenesis strategy (Stratagene, La Jolla, CA). Mutagenic primers were designed to incorporate both the desired mutation (TGC codon) and a translationally silent change to introduce a restriction site to facilitate screening. Mutations were inserted within the *LdNT1.1* open reading frame that had been ligated into the *EcoR* I site of pL2.5 *Xenopus* oocytes expression vector (*32*). Mutant clones were identified by restriction enzyme mapping, and the presence of the mutations were verified by DNA sequencing at the Oregon Health & Science University Microbiology Research Core Facility using a model 377 Applied Biosystems automated fluorescence sequencer (Perkin-Elmer). For overexpression in *L. donovani*, the cys mutant cDNAs in pL2.5 were excised with *EcoR* I and subcloned into the multicloning site of pX63NeoRI, a modified version of the leishmanial expression vector pX63Neo (*33*) where the *EcoR* I site at position 5020 was removed by site-directed mutagenesis, leaving a single *EcoR* I site in the polylinker. Mutant clones were selected by PCR screening, and mutations were confirmed again by DNA sequencing.

Cell Transfection into Leishmania and Selection of Stable Transfectants. Cys-less and single-cys mutant pX63NeoRI constructs were transfected into transport-defective Δ*ldnt1 L. donovani* promastigotes using standard electroporation conditions (*34*, *35*). Transfectants were selected and expanded in liquid medium containing 100 μg mL^{-1} of neomycin analogue G418 (Invitrogen Life Technologies, Carlsbad, CA).

cRNA Synthesis and Expression in X. laevis Oocytes. Plasmid DNAs encoding *LdNT1.1*, cys-less, and cys mutant transporters in pL2.5 were linearized with *Not* I and transcribed with T7 RNA polymerase in the presence of cap analogue [^{m7}G(5′)ppp(5′)G] using the Message Machine transcription system (Ambion Inc., Austin, TX). Microinjection of ∼25−50 ng of cRNA into *X. laevis* oocytes was performed as reported previously (*36*). Radiolabel uptake measurements were performed after 2−3 days of cRNA expression.

Transport Assays. Uptake measurements of 10 μM [^{3}H]-adenosine (American Radiolabeled Chemicals, Inc., 40 Ci/mmol) in *Xenopus* oocytes expressing wild-type *LdNT1.1*, cys-less, or cys mutant cRNAs were performed for 1 h on groups of 7−10 oocytes and analyzed by liquid scintillation counting as described (*37*).

L. donovani promastigotes expressing the cys-less and cys mutants were grown between early and mid-log phases, washed three times in phosphate-buffered saline (PBS: 138 mM NaCl, 8.1 mM Na_2HPO_4•$7H_2O$, 2.7 mM KCl, 1.5 mM KH_2PO4, pH 7.4), and resuspended in PBS to a final density of (3−4) × 10^8 parasites mL^{-1}. Nucleoside transport measurements [10 μM [^{3}H]adenosine or 3 μM [^{3}H]uridine (Moravek Biochemicals, 32.3 Ci/mmol)] were performed within the linear uptake range (40 s) per triplicate on 100 μL aliquots by the previously described oil-stop method (*3*, *38*).

MTS Modification (MTSEA, MTSES, and MTSET blocking experiments). Standard MTS treatments were performed in parallel for each substituted cys mutant and the cys-less permease that served as a negative control. For measurements of transport in *Xenopus* oocytes, adenosine uptake was assessed before and after MTS modification. Oocytes expressing the mutant transporters were preincubated for 4 min at room temperature (∼22 °C) with 2.5 mM MTS-ethylammonium (MTSEA), 1mM MTS-ethyltrimethylammonium (MTSET), and 10 mM MTS-ethyl sulfonate (MTSES) in ND96 buffer, which reflect equally reactive concentrations with free thiols in solution (*39*). Unmodified control oocytes were treated with ND96 alone. Reactions were terminated by transferring the oocytes into 1 mL of ice-cold ND96 buffer, and three subsequent washes were performed to remove the excess of the thiol-reactive agents. Before the transport activity was measured, oocytes were incubated in ND96 for 20 min and uptake assays were conducted as usual. Uptake obtained from water-injected oocytes was subtracted as background from all experimental conditions tested.

For measurements of transport in *L. donovani*, parasites were collected by centrifugation, washed three times with cold PBS, and resuspended in the same buffer ((3−4) × 10^8 parasites mL^{-1} final density). Aliquots of 100 μL (per triplicate) were incubated for 10 min with 2.5 mM MTSEA, 1 mM MTSET, and 10 mM MTSES in PBS buffer at room temperature (∼22°C). Control samples were treated with PBS alone. Adenosine and uridine uptake were subsequently assayed in triplicate on treated and control groups. For each individual mutant, the extent of the MTS reaction was quantified by comparison of the uptake in MTS-treated and nontreated samples. These values were expressed as percentages of residual activity after MTS modification.

Protection from MTS Modification. Protection experiments were performed for each accessible mutant that had an inhibition signal strength greater than 40%. To assess the effects of LdNT1.1 substrates on MTS inhibition, adenosine or uridine (5 mM final concentration) were added 30 min before adding the MTS reagents. MTS reactions were conducted in the presence of the protecting ligands and terminated either by transferring the oocytes into ice-cold ND96 buffer or by diluting the parasites (10-fold) in ice-cold PBS. Subsequent washes and uptake assays were performed as described above. Control samples of oocytes or parasites received vehicle only. Additional controls were performed by incubating the samples with either 5 mM adenosine or uridine without subsequent exposure to the MTS derivatives (data not shown). Results were expressed as normalized adenosine/uridine uptake after MTS modification, either in the presence or in the absence of the protecting ligand.

RESULTS

Generation of the Cys-Less Permease and of Mutants Containing Single Cysteine Residues Along Predicted TMD5 of the LdNT1.1 Transporter. Previous genetic studies on the LdNT1.1 permease from *L. donovani* (*9*) suggested that TMD5 (Figure 1A) is likely to be a component of the substrate translocation pathway through LdNT1.1. This transmembrane segment is predicted to form an amphipathic α-helix with the substrate-specificity determinant G_{183} located on the hydrophilic face (Figure 1B). To explore directly the

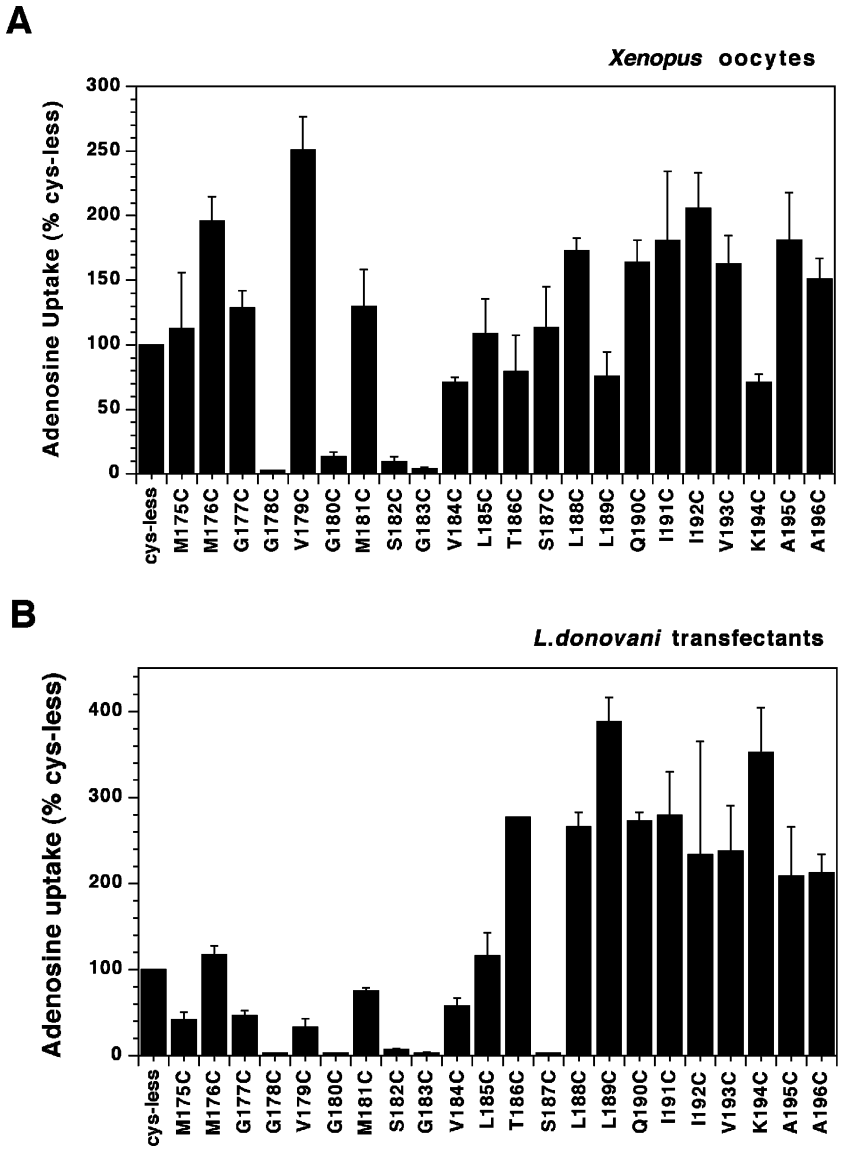

FIGURE 2: Functional expression of single-cysteine-substituted LdNT1.1 mutants. Results are shown for relative rates of [^{3}H]adenosine transport in (A) *Xenopus* oocytes and (B) Δ*ldnt1* transfectant parasite lines expressing LdNT1.1 cys-less and single-cys mutants. [^{3}H]-Adenosine [(A) 10 μM, 1 h; (B) 1 μM, 40 s] uptake was measured for the indicated times at room temperature (~22 °C). Transport results are expressed as percentages of the LdNT1.1 cys-less transport measured and are means ± SD for four to five independent experiments. There is no SD value for the cys-less samples because the average cys-less uptake value for each of the independent experiments was defined as 100%.

potential role of TMD5 in mediating substrate translocation, we have employed the substituted-cysteine accessibility method (SCAM) (*29*), a technique that was specifically developed to identify regions of membrane proteins that interact with ligands. Initially, a mutant LdNT1.1 protein in which all five native cysteine residues (C_{36}, C_{143}, C_{144}, C_{260}, C_{337}) were replaced with alanines was constructed by means of oligonucleotide-mediated site-directed mutagenesis (Figure 1A). The cys-less permease exhibited transport activity (20−25 pmol h^{-1} per oocyte) at levels comparable to that of the wild-type protein (30−35 pmol h^{-1} per oocyte), indicating that none of the endogenous cysteine residues are critical for function. This cys-less mutant was used as a background template to prepare 22 site-directed mutant constructs in which single cysteine residues were individually introduced from positions 175−196, embracing the entire predicted TM helix 5.

Functional Expression of Single-Cysteine-Substituted LdNT1.1 Mutants (cys Mutants) in Xenopus Oocytes and L. donovani Δ*ldnt1 Null Mutant.* The ability of each cys mutant to mediate ligand translocation was assessed by conducting [^{3}H]adenosine uptake assays. All mutants were expressed both in *Xenopus* oocytes and in the Δ*ldnt1* cell line (kindly provided by Dr. B. Ullman, Department of Biochemistry and Molecular Biology, Oregon Health & Science University), which was obtained by deleting both copies of the clustered *LdNT1.1* and *LdNT1.2* genes (*3*) from wild-type *L. donovani* by targeted gene replacement (*40*) and is consequently deficient in adenosine-pyrimidine transport. Results of uptake assays using each cys mutant are shown in Figure 2A,B as a percentage of the LdNT1.1 cys-less uptake value. Most of the cysteine substitutions were well tolerated, thereby demonstrating that these mutations do not induce severe perturbations in the structure of the transporter or in translocation. However, a few positions ($G_{178}C$, $G_{180}C$, $S_{182}C$, $G_{183}C$) resulted in a loss of adenosine uptake regardless of the expression system examined, whereas $S_{187}C$ only abrogated transport activity when expressed in *L. donovani*

Table 1: Effect of MTS Reagents on [^{3}H]Adenosine Uptake in *Xenopus* Oocytes and *L. donovani* Parasites Expressing the Cys-Less and the Functional Single-Cys Mutants[a]

	X. laevis oocytes			*L. donovani* parasites		
mutant	MTSEA	MTSES	MTSET	MTSEA	MTSES	MTSET
cys-less	89.7 ±14.74	94.4 ± 10.03	106.1 ± 11.44	**60.5 ± 10.70**	98.5 ± 18.90	83.1 ± 2.82
$M_{175}C$	83.3 ± 4.37	75.9 ± 6.5	100.6 ± 11.38	65.3 ± 10.60	68.3 ± 6.50	68.5 ± 6.36
$M_{176}C$	**30.3 ± 3.42**	70.0 ± 8.49	100.5 ± 4.95	**25.3 ± 1.04**	100.5 ± 0.71	98.0 ± 2.82
$G_{177}C$	65.7 ± 3.25	66.9 ± 4.10	91.6 ± 8.69	72.7 ± 9.90	65.6 ± 3.51	108.8 ± 2.25
$V_{179}C$	86.7 ± 0.57	101.4 ± 5.23	95.6 ± 2.25	62.0 ± 7.23	93.3 ± 14.17	92.6 ± 13.66
$M_{181}C$	73.0 ± 4.57	73.3 ± 3.20	85.4 ± 5.05	90.5 ± 6.10	94.5 ± 10.19	115.2 ± 9.33
$V_{184}C$	91.3 ± 5.65	87.1 ± 6.79	100.9 ± 2.03	81.0 ± 4.24	86.5 ± 6.26	158.6 ± 12.85
$L_{185}C$	76.3 ± 2.04	87.9 ± 2.59	82.0 ± 1.41	98.0 ± 8.49	79.3 ± 5.92	132.5 ± 13.64
$T_{186}C$	**22.1 ± 6.80**	**30.3 ± 8.32**	94.4 ± 18.50	**22.5 ± 14.80**	**60.0 ± 2.83**	115.0 ± 4.24
$S_{187}C$	**20.4 ± 2.28**	105.6 ± 9.60	**60.2 ± 8.61**	b	b	b
$L_{188}C$	78.4 ± 2.31	84.1 ± 1.33	85.7 ± 3.39	79.5 ± 0.71	121.5 ± 12.90	129.0 ± 14.32
$I_{189}C$	67.0 ± 5.32	97.5 ± 9.34	96.4 ± 6.30	108.5 ± 12.0	118.0 ± 9.56	123.0 ± 2.83
$Q_{190}C$	**55.1 ± 7.60**	100.1 ± 17.47	**35.4 ± 4.38**	**12.4 ± 1.60**	**30.5 ± 2.19**	**15.4 ± 0.71**
$I_{191}C$	103.0 ± 7.55	97.2 ± 5.90	111.0 ± 1.41	81.0 ± 9.90	109.0 ± 8.30	98.0 ± 7.07
$I_{192}C$	111.7 ± 5.89	121.8 ± 12.33	97.2 ± 4.32	73.3 ± 15.08	85.5 ± 16.40	163.0 ± 26.7
$V_{193}C$	**13.4 ± 1.24**	108.6 ± 8.59	92.0 ± 4.24	**14.0 ± 3.24**	**60.5 ± 2.02**	103.5 ± 13.30
$K_{194}C$	95.5 ± 12.94	97.6 ± 18.59	**53.5 ± 5.43**	**55.0 ± 7.02**	107.5 ± 10.60	103.0 ± 15.21
$A_{195}C$	73.3 ± 9.81	89.8 ± 6.29	82.4 ± 12.8	116.5 ± 2.12	93.5 ± 25.00	156.2 ± 15.92
$A_{196}C$	69.2 ± 7.70	84.6 ± 8.47	76.7 ± 9.47	111.0 ± 8.80	106.7 ± 5.77	163.7 ± 3.21

[a] Data are expressed as percentages of residual activity after MTS modification by comparison of the uptake in MTS-treated and nontreated samples (for details, see Experimental Procedures). Those mutants exhibiting ≤60% residual activity (at least 40% inhibition) are indicated by bold values and were tested further with the corresponding MTS reagents in protection experiments (Figure 3). Results represent the mean ± SD of three to seven independent experiments, each performed in triplicate. [b] $S_{187}C$ mutant is nonfunctional when expressed in the Δ*ldnt1* cell line.

parasites. This evidence suggests that G_{178}, G_{180}, S_{182}, and G_{183} residues are structurally or functionally important to LdNT1.1 transport activity. Nonetheless, the observation that 18 of the 22 cys mutants (including $S_{187}C$ when expressed in *Xenopus* oocytes) retained substantial adenosine transport activity confirmed that the SCAM could be employed to monitor the accessibility of most of the substituted cysteine residues to chemical modification from the aqueous phase as well as the ability of substrates to block such modification.

Modification of Transport Activity of Cys Mutants by Methanethiosulfonate (MTS) Reagents and Substrate Protection from Modification. The solvent accessibility of the cysteine residues was investigated by measuring the ability of the mutated permeases to transport adenosine before and after modification by small, thiol-reactive MTS agents. Since the reactivity of an individual cysteine might depend on the environment surrounding it, we screened three MTS derivatives (MTSEA, MTSET, and MTSES) with varying sizes and charges to minimize any potential steric or electrostatic effect that could attenuate modification. While the larger and more highly charged MTSET and MTSES do not permeate the lipid bilayer (*11*, *41*, *42*) the smaller MTSEA has the reported ability to diffuse through the membranes and can thus react with the permease from both sides of the plasma membrane. Moreover, to provide further evidence for a pore-lining location of any substituted cysteine, we attempted to attenuate the rates of the MTS reaction by including 5 mM adenosine both before and during treatment with the modifying agents. Although all of the 18 functional single-cys mutants were assayed for [^{3}H]adenosine uptake (Table 1), only those exhibiting at least 40% inhibition after MTS treatment are shown in Figure 3. Effects at the other positions tested were not sufficiently pronounced to conclude that they were accessible to the aqueous phase. For each cys mutant, the effect of treatment with MTS reagents and protection by substrates was monitored in both the oocyte (Figure 3A) and the transfected parasite (Figure 3B) expression systems.

Measurement of adenosine uptake in MTS-treated oocytes (Figure 3A) revealed six residues ($M_{176}C$, $T_{186}C$, $S_{187}C$, $Q_{190}C$, $V_{193}C$, and $K_{194}C$) that showed significant loss of transport activity upon MTS reaction. In each series of experiments, cys-less served as a negative control, displaying no significant sensitivity toward the sulfhydryl derivatives. Five ($M_{176}C$, $T_{186}C$, $S_{187}C$, $Q_{190}C$ and $V_{193}C$) of the six sensitive mutants reported above were susceptible to reaction with the small, membrane-permeable, positively charged MTSEA. Of the residues that reacted with MTSEA, MTSET reacted with $S_{187}C$ and $Q_{190}C$ and no others. Apparently, the accessibility of $M_{176}C$, $T_{186}C$, and $V_{193}C$ might be more restricted, allowing the small and flexible MTSEA reagent but not MTSET, which is not permeable and has a bulkier side chain, to reach the residues (*42*, *43*). Moreover, in contrast to MTSEA, MTSET exposure also modified $K_{194}C$ activity. In this case, the lack of an observable effect with MTSEA might result from the modification on the cysteine being too small to interfere with substrate binding. Analogous experiments performed with the negatively charged MTSES only blocked $T_{186}C$ transport. Positions unaffected by MTSES treatment might be either inaccessible to the negatively charged thiol reagent or their modification might have no effect on function.

Measurement of adenosine uptake in MTS-treated parasites (Figure 3B) revealed that the global pattern of modification was conserved across both expression systems. However, the transfected cys-less control was reproducibly inhibited up to 40% after MTSEA exposure in more than 10 individual experiments. It remains unclear why cys-less is partially sensitive to MTSEA, especially in light of the fact that most of the nonsensitive cys mutants examined were not significantly inhibited by this reagent (see the $L_{185}C$ unaffected mutant in Figure 3C as a representative example). On the other hand, since individual cysteine replacement of S_{187} resulted in a *L. donovani* transport-deficient phenotype, this position could not be assessed by SCAM. In summary, five

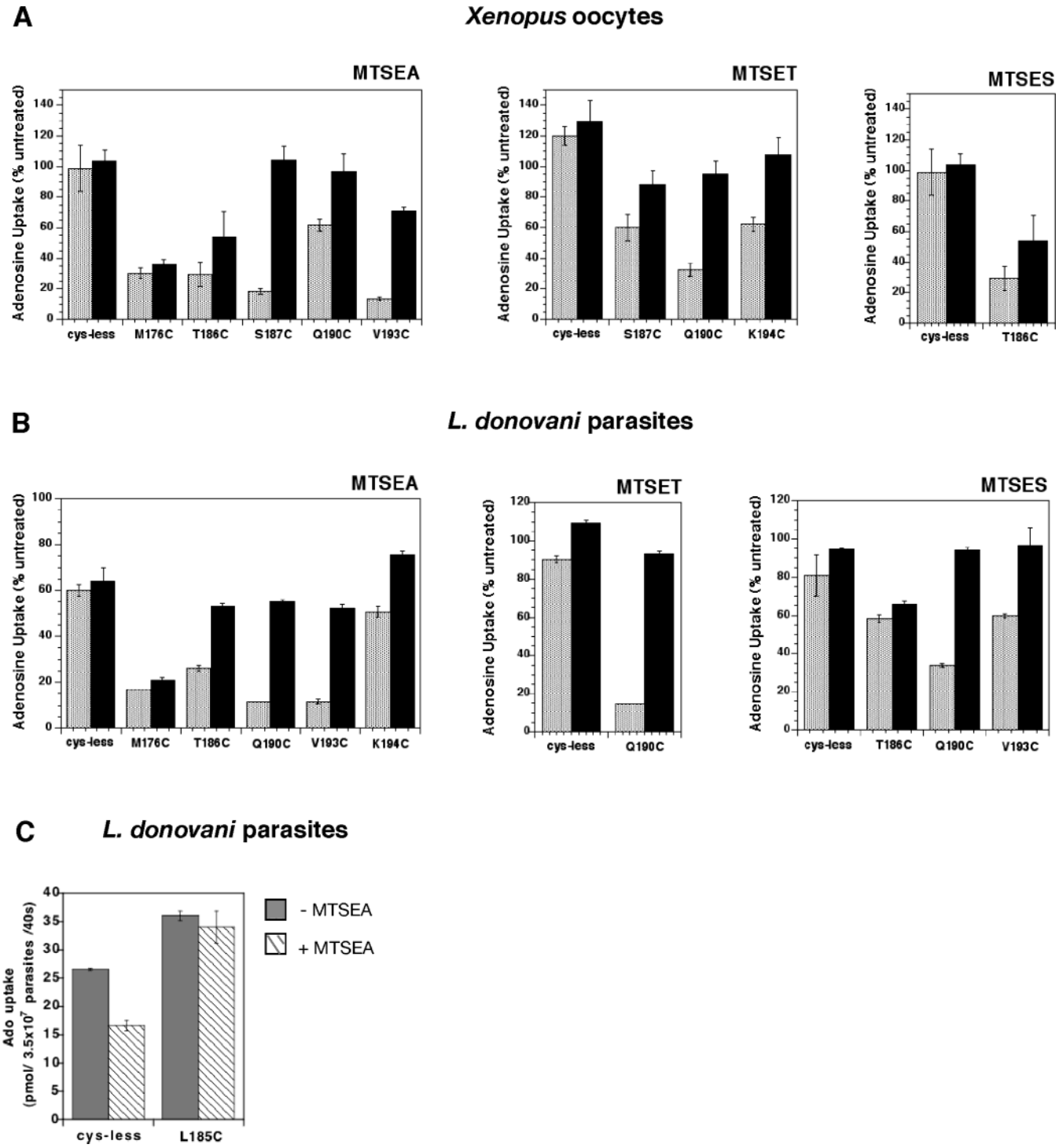

FIGURE 3: Effects of MTS treatment and adenosine protection on adenosine uptake by single-cys mutants. (A) Microinjected *Xenopus* oocytes and (B) transfected *L. donovani* cell lines expressing cys-less and the indicated cys mutants were incubated for 4−10 min at room temperature (~22 °C) with 2.5 mM MTSEA, 1 mM MTSET, and 10 mM MTSES in the presence or absence of 5 mM adenosine. [^{3}H]-Adenosine uptake assays were subsequently conducted [(A) 10 μM, 1 h; (B) 1 μM, 40 s] at ~22 °C, as described in Experimental Procedures. For each mutant, uptake values were normalized to the uptake observed in the absence of MTS reagents, either in the presence (black bars) or the absence (gray bars) of 5 mM adenosine. Data shown correspond to a representative experiment of five to seven independent determinations [(A) each using 5−10 oocytes per experimental group or (B) done in triplicate] that gave virtually the same results. Figure C shows the adenosine uptake values (pmol/3.5 × 10^7 parasites/40 s) for the cys-less transfectant and a representative cys mutant ($L_{185}C$) whose activity remains unaffected both in the presence (crosshatched) and in the absence (gray bars) of the MTSEA reagent. Results represent the mean ± SD of five independent experiments, each performed in triplicate.

residues ($M_{176}C$, $T_{186}C$, $Q_{190}C$, $V_{193}C$, and $K_{194}C$) showed significant loss of adenosine uptake upon MTS reaction in the parasite expression system. All of them were inhibited by MTSEA, whereas only $Q_{190}C$ was modified by MTSET and $T_{186}C$, $Q_{190}C$, and $V_{193}C$ resulted in a loss of activity by MTSES.

Notably, 5 mM adenosine significantly protected all of the sensitive cys mutants except $M_{176}C$ from the inhibitory

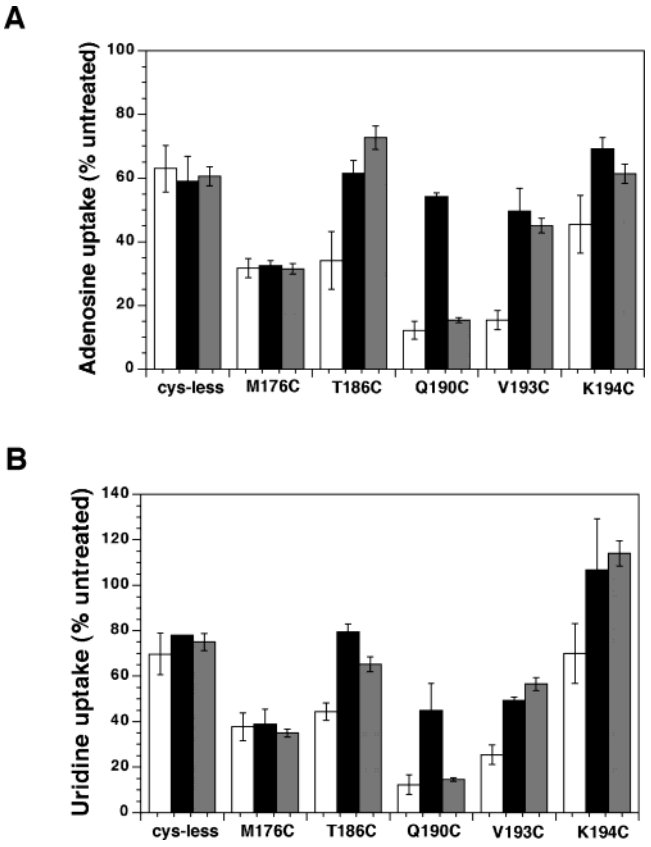

FIGURE 4: Effects of MTSEA modification and substrate protection on adenosine and uridine uptake by single-cys mutants. *L. donovani* Δ*ldnt1* cell lines expressing cys-less and the indicated cys mutants were incubated for 10 min at room temperature (~22 °C) with 2.5 mM MTSEA in the presence or absence of 5 mM adenosine or uridine. (A) [^{3}H]Adenosine (1 μM) and (B) [^{3}H]uridine (3 μM) uptake assays were subsequently performed in parallel for 40 s at ~22 °C, as described in Experimental Procedures. For each mutant, uptake values were normalized to the uptake observed in the absence of MTSEA, either in the presence of 5 mM adenosine (black bars) or 5 mM uridine (gray bars) or in the absence of any substrate (white bars). Results represent the mean ± SD of seven independent experiments, each performed in triplicate.

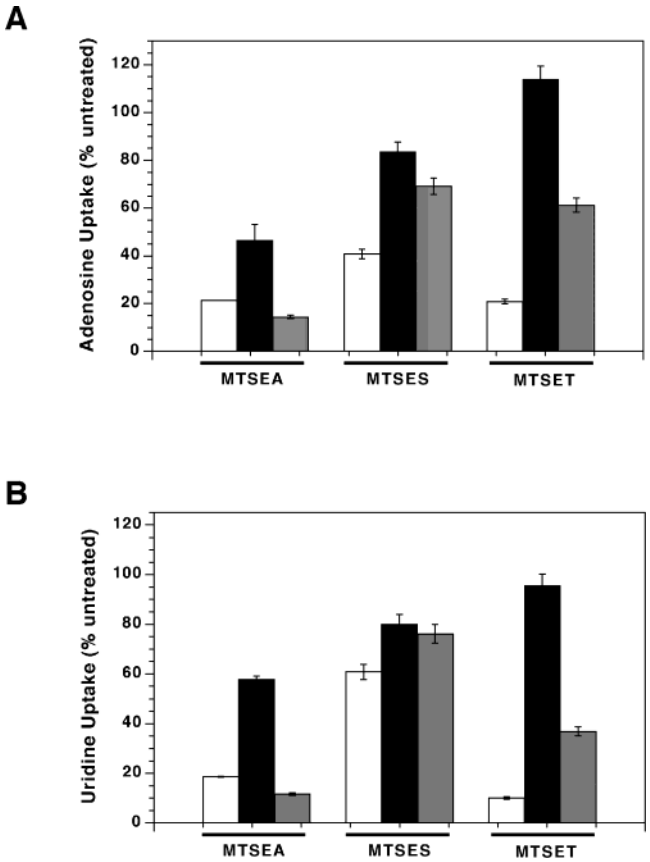

FIGURE 5: MTS modification profile of $Q_{190}C$ mutant. The Δ*ldnt1* null mutant cell line expressing the $Q_{190}C$ substitution was incubated for 10 min at room temperature (~22 °C) with 2.5 mM MTSEA, 1 mM MTSET, and 10 mM MTSES in the presence or absence of 5 mM adenosine or uridine, as described before. (A) [^{3}H]adenosine (1 μM) and (B) [^{3}H]uridine (3 μM) uptake assays were performed in parallel for 40 s at ~22 °C, and uptake values were normalized to the uptake observed in the absence of MTS reagents, either in the presence of 5 mM adenosine (black bars) or 5 mM uridine (gray bars) or in the absence of any substrate (white bars). Representative results from one of three independent experiments, each done in triplicate, are shown.

effects of MTS-binding, regardless of the expression system analyzed. Even though the extent of protection substantially varied among mutants, the ability of the substrate to significantly retard loss of activity provided further evidence for a pore-lining location of the protected residues.

The cysteine accessibility and substrate protection results are summarized in a helical wheel diagram (Figure 1B), where residues that are affected by MTS modification are circled and those that are protected by 5 mM adenosine are indicated by arrows. As predicted for an amphipathic α-helix that is part of an aqueous translocation pathway, all of the sensitive and substrate-protected residues clustered together along the hydrophilic face of the helix proposed for TMD5.

Glutamine 190 Interacts Differently with Two Different Substrates. Further analysis of the effect of transportable substrates on the protection of the reactive cys mutants against modification by MTSEA revealed an intriguing result. Protection from MTSEA reaction by saturating amounts (5 mM) of either adenosine or uridine was monitored in parallel using the *L. donovani* expression system. Even though the inhibition by uridine resembled that observed by adenosine for most cysteine substitutions (Figure 4A), MTSEA modification at position 190 ($Q_{190}C$) was blocked by adenosine but not by uridine when [^{3}H]adenosine uptake was measured. This result suggests that adenosine, but not uridine, interacts with $Q_{190}C$. We were also interested in determining whether the inability of uridine to protect $Q_{190}C$ from the modifying effects of MTSEA extended to [^{3}H]uridine uptake capability. Of the 22 cys mutants within TMD5, 17 were able to accumulate 3 μM [^{3}H]uridine above background levels. Notably, the same transport-incapacitating mutations that were found to impair adenosine uptake ($G_{178}C$, $G_{180}C$, $S_{182}C$, $G_{183}C$, and $S_{187}C$) also resulted in a loss of uridine influx (data not shown). Figure 4B presents the effects on [^{3}H]-uridine uptake of treating the $M_{176}C$, $T_{186}C$, $Q_{190}C$, $V_{193}C$, and $K_{194}C$ cys mutants with MTSEA, either in the presence or absence of 5 mM adenosine or uridine. The inhibition profile observed was virtually identical to the one inferred for the adenosine transport (Figure 4A).

Protection experiments for $Q_{190}C$ were also performed using MTSET and MTSES modification (Figure 5). For inhibitions performed with MTSET, protection with uridine preserved at least some level of either [^{3}H]adenosine or [^{3}H]-uridine uptake, although not to the extent of the protective effect exhibited by adenosine. Modification of $Q_{190}C$ with

MTSES revealed similar protection of transport activity by both adenosine and uridine, although the inhibition of [^{3}H]-uridine uptake by MTSES was relatively small (~20−40% in several independent experiments). Thus, the strong differential protection of $Q_{190}C$ by adenosine compared to uridine appears to be restricted to modification by MTSEA.

DISCUSSION

Cysteine Scanning Mutagenesis of TMD5. In the present SCAM analysis of TMD5 in LdNT1.1, 18 out of 22 single-cys mutants within the putative amphipathic α-helix were capable of mediating substrate uptake above background levels. The fact that three of the four glycines within TMD5 resulted in loss of transport activity when mutated to a cysteine is not surprising, since the mutation of glycines within TM helices often impairs protein function. Glycine residues are known to impart conformation flexibility (*44*) and to contribute to helical packing in membrane proteins (*45*, *46*). In addition, G_{180} and G_{183} (positions G_{179} and G_{184} for the human ENT, hENT1) are residues that are highly conserved across equilibrative nucleoside permeases from several species (*47*), suggesting that they fulfill a crucial role in the function of these transporters. Indeed, mutations of G_{179} and G_{184} to leucine, cysteine, or valine have been found to abolish transport activity and tubercidin sensitivity of hENT1 (*47*). These results are consistent with our previous observation that a $G_{183}D$ substitution in LdNT1.1 also abrogates function and tubercidin cytotoxicity (*9*). We do not know whether the nonfunctional cysteine mutants traffic correctly to the plasma membrane or are retained intracellularly, as there is no antiserum available that reacts with LdNT1.1. However, this question is largely irrelevant to the determination of the substrate translocation pathway.

The remaining functional cys mutants were characterized in both the homologous *L. donovani* and the heterologous *Xenopus* oocyte expression systems. The dual characterizations largely confirm each other, thus further reinforcing our conclusion that TMD5 of LdNT1.1 constitutes part of the substrate translocation pathway. Specifically, the $M_{176}C$, $T_{186}C$, $Q_{190}C$, $V_{193}C$, and $K_{194}C$ mutations were susceptible to modification by MTS reagents in both expression systems, and all of them except $M_{176}C$ could be protected by the substrate adenosine.

One discrepancy we observed is that some cysteine substitutions (e.g., $V_{179}C$) that cause partial inhibition of transport activity in *L. donovani* result in activation in *Xenopus* oocytes (Figure 2A,B). Although we do not know the reasons for the differential effects of these substitutions in the two systems, we emphasize that these divergences are unimportant for the identification of the substrate translocation pathway. This is because SCAM identifies the pathway by determining the *relative* transport activity of each residue before and after chemical modification rather than the absolute activity of the unmodified cys mutant. A second difference that emerged between both expression systems is that the $S_{187}C$ mutant is functional in transport in the oocyte but inactive in the parasite (Figure 2A, B). While we also do not know the reason for this disparity, the results in the oocyte model where the mutant is active are useful and suggest that residue 187 is also part of the translocation pathway, consistent with its position on the hydrophilic face of TMD5. We could speculate thus that the $S_{187}C$ substitution might be sufficient to abrogate transport activity by either disrupting the interaction with substrates or destabilizing the interaction of substrates with the neighboring residues in the parasite membrane. However, when expressed in the heterologous environment of the oocyte membrane, the mutant protein might acquire a slightly different conformation, leading to a transporter still active although susceptible to modification by the MTS reagents.

Modification of Substituted Cysteine Residues by MTS Reagents. Modification of a substituted cysteine residue by an MTS reagent and the accordant loss of transport activity constitutes evidence that the residue is accessible to the aqueous phase, as would be expected for a component of the substrate translocation pathway (*10*). This conclusion follows from the fact that only the ionized form of the cysteine sulfhydryl reacts with MTS reagents (*48*), and this ionized sulfhydryl is very unstable within the lipid bilayer. Helical wheel analysis of the results of the MTS modification experiments on LdNT1.1 (Figure 1B) revealed that the six residues accessible to the MTS derivatives cluster together along the hydrophilic face of the putative α-helix predicted for TMD5. Amphipathic helices may be components of substrate translocation pathways in many transporters, with the hydrophilic residues forming specific interaction with substrates (*10*, *22*, *49*). Of note, this helical face encompasses G_{183}, which has been hypothesized to lie within the aqueous translocation pore in LdNT1.1, given its demonstrated role in determining substrate specificity (*9*). We anticipate that other TMDs of ENTs will also likely form components of the substrate binding sites and translocation pathway.

Substrate Protection of Sulfhydryl Modification by MTS Reagents. Further evidence for a pore-lining location was provided by substrate protection assays. The binding of substrate protected five of the six sensitive residues from modification, although the extent of the protection varied considerably among the mutants and reagents tested. Globally, MTSET and MTSES reactions resulted in protection percentages close to 100%, whereas MTSEA modifications were more poorly inhibited. One possible explanation is that the permeable MTSEA can approach the cysteines introduced from both sides of the membrane, whereas in the case of the impermeable MTSES and MTSET, the approach has to be exclusively from the outside (*11*, *41*, *42*) and thereby more easily blocked by substrate competition. The lack of protection observed for $M_{176}C$ may be attributable to its position at one extreme of the helix that likely locates this residue at the margin of the binding site. Similarly, the lower degree of MTS-associated inhibition imputed to $K_{194}C$, compared to modification at most other positions, although protected by substrate, might be explained by its location at the other extreme of the helix. Thus, we might speculate that rather than being central to ligand translocation or recognition, $M_{176}C$ and possibly $K_{194}C$ react with specific MTS reagents and lead to reduction in transport activity due to their proximity to the binding crevice.

To gain additional insights on the substrate binding domains, protection assays of the sensitive cys mutants were tested with both adenosine and uridine. A unique finding in the present study was the differential protection of the highly sensitive $Q_{190}C$ mutant from MTSEA modification by adenosine but not by uridine. A possible explanation invokes

Q_{190} as a potential contact point or binding determinant for adenosine but not uridine recognition. Thus, while it is conceivable that binding of adenosine physically prevents the access of any of the MTS reagents to $Q_{190}C$, the absence of protection by uridine might relate to a distinct but likely overlapping binding site that would allow the access of the small and flexible MTSEA derivative but restrict, albeit partially, the binding of the bulkier MTSET and MTSES molecules (Figure 5). Although these studies are too preliminary to speculate further on the specific role of Q_{190} in the translocation mechanism, they are consistent with the notion of Q_{190} interacting with adenosine but not uridine. Moreover, it is noteworthy that Q_{190} is located in the same rotational plane of the helix as the substrate-specificity determinant G_{183}, and future assays using site-directed mutagenesis approaches should highlight whether Q_{190} constitutes a new determinant of substrate selectivity through the LdNT1.1 permease.

Comparison to Studies on Other ENT Family Members. Collectively, the inferred accessibility pattern for LdNT1.1 complements previous but more limited studies reported for other members of the ENT family. Thus, by using chimeras between the human hENT1 and hENT2 permeases, the region encompassing TM-spanning domains 3−6 has been implicated in binding of competitive inhibitors of these permeases (*50*). Furthermore, modification of C_{140} in TMD4 of rENT2 results in loss of transport function, but the modification can be protected by substrate, strongly suggesting that TMD4 forms part of the substrate translocation pathway through the rat transporter (*51*). Mutational dissection of G_{179} of hENT1 (*47*) signified a role for this residue in TMD5, albeit potentially an indirect one, in ligand translocation and interaction with the competitive inhibitor NBMPR. In addition, conserved residues within TMD8 of the LdNT2 inosine/guanosine transporter recently suggested that TMD8 also contributes to the ENT translocation mechanism and that intrahelical interactions within TMD8 are important determinants of transporter stability (*52*). Our results extend these findings by providing direct evidence that the hydrophilic face of TMD5 interacts with substrate and contributes to the translocation pathway of the permease. These results imply that a similar structural paradigm may exist for ENT family members in organisms ranging from *L. donovani* to the human.

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