Aspartate 19 and Glutamate 121 Are Critical for Transport Function of the myo-Inositol/H\(^{+}\) Symporter from *Leishmania donovani*

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The protozoan flagellate *Leishmania donovani* has an active myo-inositol/proton symporter (MIT), which is driven by a proton gradient across the parasite membrane. We have used site-directed mutagenesis in combination with functional expression of transporter mutants in *Xenopus* oocytes and overexpression in *Leishmania* transfectants to investigate the significance of acidic transmembrane residues for proton relay and inositol transport. MIT has only three charged amino acids within predicted transmembrane domains. Two of these residues, Asp\(^{19}\) (TM1) and Glu\(^{121}\) (TM4), appeared to be critical for transport function of MIT, with a reduction of inositol transport to about 2% of wild-type activity when mutated to the uncharged amides D19N or E121Q and 20% (D19E) or 4% (E121D) of wild-type activity for the conservative mutations that retained the charge. Immunofluorescence microscopy of oocyte cryosections showed that MIT mutants were expressed on the oocyte surface at a similar level as MIT wild type, confirming that these mutations affect transport function and do not prevent trafficking of the transporter to the plasma membrane. The proton uncouplers carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone and dinitrophenol inhibited inositol transport by 50–70% in the wild-type as well as in E121Q, despite its reduced transport activity. The mutant D19N, however, was stimulated about 4-fold by either protonophore and 2-fold by cyanide or increase of pH 7.5 to 8.5 but inhibited at pH 6.5. The conservative mutant D19E, in contrast, showed an inhibition profile similar to MIT wild type. We conclude that Asp\(^{19}\) and Glu\(^{121}\) are critical for myo-inositol transport, while the negatively charged carbonylate at Asp\(^{19}\) may be important for proton coupling of MIT.

In kinetoplastid protozoa such as *Leishmania* myo-inositol plays an especially important role as the precursor for various inositol phospholipids found in the great majority of surface molecules in these parasites. These include glycosyl-phosphatidylinositol-anchored surface proteins such as the major surface glycoprotein, gp63 (1, 2), or abundant inositol-containing glycolipids such as lipophosphoglycan (3) and glycoinositolphospholipids (4). Several of these surface molecules are involved in the invasion of macrophages in the mammalian host or the attachment of the parasite to the epithelium of the insect midgut. Strikingly, these glycosyl-phosphatidylinositol-anchored surface proteins and glycosyl-phosphatidylinositol-related glycolipids are several orders of magnitude more abundant on the surface of these parasites than in the mammalian host (5).

In a previous study (6), we cloned a myo-inositol transporter (MIT) from the parasitic protozoan *Leishmania donovani*, the causative agent of fatal visceral leishmaniasis and an increasing health problem as an opportunistic infection in immunocompromised individuals (7, 8). MIT shows significant amino acid similarity with two inositol transporters from the yeast *Saccharomyces cerevisiae*, ITR1 and ITR2 (9), and is a member of the 12-transmembrane domain sugar transporter superfamily (10, 11). Functional expression of MIT in *Xenopus laevis* oocytes has revealed that it is an active myo-inositol/proton symporter driven by a proton gradient across the cell membrane (12). Subsequently, voltage clamp recording was used to characterize a 1:1 stoichiometry of transport with an ordered binding of a proton followed by a molecule of myo-inositol and subsequent transport of both molecules across the membrane (13). Finally, immunolocalization studies revealed that this transporter is expressed on the plasma membrane, flagellar pocket, and flagellum of the parasite (12).

Many transport processes in these flagellates are thought to be proton-driven, and MIT thus presents an excellent model for the study of one such proton symporter in *Leishmania*. A plasma-membrane proton ATPase has been identified in *L. donovani* (14), which can provide the transmembrane proton gradient for active secondary transporters. Knowledge of the function of protozoan transporters is still very limited, but from studies in prokaryotes, proton relay across cell membranes is thought to involve negatively charged amino acids within membrane-spanning domains (15). Two well studied examples are the lactose/H\(^{+}\) symporter lac permease of *Escherichia coli* (16) and bacteriorhodopsin of halobacteria (17). Extensive mutagenesis of lac permease has revealed only four amino acids essential for transport, and all are thought to be charged transmembrane residues (18). In addition, studies with the melibiose permease from *E. coli* (19) have defined a role for transmembrane aspartate residues in interaction with the counterions H\(^{+}\) or Na\(^{+}\).

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**Materials and Methods**

**Parasite Culture and MIT Expression in Oocytes—**Promastigotes of the *L. donovani* DI-700 clone (Sudan strain 15; Ref. 20) were cultured at 27 °C in Dulbecco’s modified Eagle’s medium, adapted for *Leishmania* (DMEM-L medium; Ref. 21), containing 0.9% bovine serum albumin (fraction V; Sigma) to replace fetal calf serum. Defolliculated stage X. laevis oocytes were microinjected with 8–10 ng (40 nl) of capped MIT cRNA, which was transcribed in vitro from linear MIT.pL2–5 template DNA by T7 RNA polymerase (Promega) as described previously (22). Injected oocytes were incubated for 3–5 days at 16 °C and 80 rpm shaking in ND-96 buffer (22) supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, and 50 mM theophylline.

**Site-directed Mutagenesis—**Oligonucleotide-directed, site-specific in vitro mutagenesis was a modification of the protocol of Titus (24). Single-stranded DNA mutagenesis template was prepared from MIT.pL2-5 plasmid as described (25). Briefly, 0.06 pmol of single-stranded DNA mutagenesis template was annealed with 1.7 pmol of phosphorylated mutagenic oligonucleotide (Table I) for 75 °C, gradually cooled down to 45 °C within 30 min and to room temperature within 10 min. Mutant strand synthesis by primer extension and ligation was carried out for 90 min at 37 °C using 8 units of T4 DNA polymerase (Promega) and 3 units of T4 DNA ligase (Promega). One of these mutagenesis reactions (about 10 ng of template DNA) was transformed into the *E. coli* E. coli ElS1301 mut S strain to suppress in vitro mismatch repair (26) and amplified in liquid culture under ampicillin selection. Subsequently, 1 ng of mut S-derived plasmid DNA was transformed into *E. coli* XL-1 Blue cells and individual colonies were selected from agar plates. Mutagenic oligonucleotides were designed to generate the appropriate aspartate or glutamate mutation and to introduce simultaneously a silent restriction endonuclease site alteration (Table I). Mutant clones were identified by restriction enzyme mapping with a yield of 20–50% of mutants. The presence of the mutation was later verified by DNA sequencing using the dideoxy chain termination method (27). For the mutations affecting inositol uptake (D19N, D19E, E121Q, E121D), the entire mutant gene was sequenced to confirm the introduction of the desired mutation and the absence of any other sequence alterations.

**Plasmid Constructs and Transformation into Leishmania—**For expression of the MIT gene in oocytes, MIT (formerly designated D1; Ref. 6) was subcloned into the Xenopus expression vector pl2-5 (Ref. 28; kindly provided by Dr. Susan Amara, Vollum Institute) as reported by Drew et al. (12) to produce the MIT.pL2-5 plasmid. For overexpression of the MIT gene in *L. donovani* promastigotes, the MIT.HindIII-HindIII insert of MIT.pL2-5 was subcloned into the Leishmania expression vector pX-H, derived from vector pX (Ref. 29; kindly provided by Dr. Stephen Beverley, Harvard Medical School) by introducing a HindIII site into the BamHI site of pX, to produce the MIT.pX-H plasmid. Transfection of the MIT.pX-H plasmid into *L. donovani* promastigotes was performed by electroporation as described previously (30), and transfectants were selected in liquid medium containing 200 μg/ml neomycin analog G418 (Life Technologies, Inc.).

**Transport Assays—**For transport assays in *Leishmania* and oocytes, myo-[2-\(^{3}H\)inositol (specific activity of 21 Ci/mmol; NEN Life Science Products) was utilized. Promastigotes from middle to late log phase *L. donovani* culture, transfected with MIT.pX-H, were washed twice in phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS. Transport was measured at 25 °C and initiated by adding 100 μl of cells (5 × 10^7) to 100 μl of radiolabeled myo-inositol at 50 μM final concentration in PBS. At various time points between 10 and 120 s, transport was stopped by spinning the cells through an oil cushion in a microcentrifuge tube, followed by immediate snap freezing of the tube in a dry ice/ethanol bath. The tip of the tube with the frozen cell pellet was then clipped off into 250 μl of 1% SDS, mixed with 2 ml of EcoLume (ICN, Costa Mesa, CA), and analyzed by liquid scintillation counting. The initial myo-inositol uptake rate was determined by linear regression analysis from the linear uptake range for the various transfectants.

Transport measurements in *Xenopus* oocytes were performed at room temperature and initiated by adding three to four oocytes to 300 μl of radiolabeled myo-inositol (50 μM to 3 μM final concentration) in ND-96 buffer. After 30 min of incubation, the oocytes were washed three times in 2 ml each of ND-96 buffer. Subsequently, each oocyte was individually solubilized in 250 μl of 1% SDS and analyzed by liquid scintillation counting in EcoLume as described above. Water-injected oocytes served as control for MIT-specific inositol uptake determination. For inhibitor studies, propanolamines or other potential inhibitors (applied from an ethanol stock solution) were preincubated with the oocytes for 10 min prior to the initiation of uptake assays, and cells incubated with 1% ethanol served as control. Statistical analysis of the data was performed by the paired sample t test, and all p values are two-tailed (31). For the substrate saturation kinetics, K_\text{m} and V_\text{max} values were determined by non-linear regression fit of the data to the Michaelis-Menten equation, employing the Levenberg-Marquardt algorithm (Kaleidagraph program, Synergy Software) (22).

**Confocal Immunofluorescence Microscopy—**Antibody against the 136-amino acid hydrophilic COOH-terminal domain of MIT (MIT-COOH) was produced against an MIT-COOH glutathione S-transferase fusion protein (12). For MIT immunolocalization in oocyte cytosol, MIT-specific-ir oocytes (from wild-type or mutant) were fixed in 2% formaldehyde/PBS for 2 h at room temperature, infiltrated with 20% sucrose/PBS overnight at 4 °C, and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek U.S.A., Torrance, CA) at least 5 h at room temperature (32). Embedded samples were rapidly frozen in dry ice, and semithin cryosections (8–10 μm) were cut on a cryostat, mounted on poly-l-lysine-coated slides, dried, and stored at −20 °C. Subsequently, MIT protein was immunolocalized in oocyte sections blocked with 5% BSA in PBS for 10 min, followed by a 1-h incubation with MIT-COOH rabbit antiserum (diluted 1:100 in 1% BSA/PBS) and Texas Red-conjugated secondary antibody (Molecular Probes, Eugene, OR; diluted 1:500 in 1% BSA/PBS) for 30 min. Oocyte sections were examined with a Leica confocal laser-scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a Leica X.63 oil immersion lens as described previously (30). All oocytes in Fig. 4 came from the same batch, and for each mutant, oocytes from the same microinjection were tested for mutant-specific inositol uptake prior to the embedding procedure.

**Results**

**Inositol Uptake in MIT Mutants—**As shown in Fig. 2, alteration of either Asp\(^{19}\) (TM1) or Glu\(^{121}\) (TM4) from the charged carboxylate to the uncharged amide reduced transport activity to about 2% of wild-type activity when the mutants were expressed in *Xenopus* oocytes. For all measurements in oocytes, MIT-specific inositol uptake was determined by subtracting the values of water-injected control oocytes from the same batch for each experiment, and the resulting D19N-specific and E121Q-specific inositol uptake, although low, was significantly different from the background level of water-injected oocytes, with p < 0.01 and p < 0.02, respectively (paired sample t test). The mutant D32N in extracellular loop 1 served as control and did not show significant differences from wild-type.
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| MIT       | Mutagenic primer | Nucleotide | Nucleotide change | Restriction site alteration |
|-----------|------------------|------------|------------------|---------------------------|
| D19N      | 5'-'GAT:GAC GCA+ C CGT | 730 | G → A | DrdI site lost         |
| D19E      | 5'-'GAT:GAC GCA+ C CGT | 732 | C → A | DrdI site lost         |
| D32N      | 5'-'GAA GCC:GAA GT+ C TTC:TT CAT CTG GAA-3' | 779 | G → A | New XnuI site          |
| E95Q      | 5'-'CAC GAC CAC CAC TT:GG CAC+ ATT TGG GGC-3' | 958 | G → C | BsiYI site lost        |
| E121Q     | 5'-'CCG CCA TGT TAG TT:GG CAC CAG GTA CAC-3' | 1036 | G → C | New CfoI site          |
| E121D     | 5'-'AGT TAG GTG CCG TGC CAG GTA CAC:AC ATG GGT GGC-3' | 1023, 1038 | A → T, A → C | BsrI site lost        |

Table I: Mutations introduced into MIT

Italics indicate nucleotides replaced in mutants. The affected codon is underlined. Colonics indicate beginning and end of the affected restriction endonuclease recognition sequence with the cutting site marked by an asterisk. The mutagenic primer is in antisense orientation and was annealed to single-stranded template DNA of sense orientation. Nucleotide position is numbered in sense orientation according to Langford et al. (6).

**Fig. 2. myo-Inositol uptake in MIT mutants expressed in X. laevis oocytes or overexpressed in Leishmania parasites.** For uptake studies in oocytes (left), in vitro transcribed cDNA was micro-injected at a concentration of 8–10 ng/oocyte. After 3 days of expression, uptake of myo-[\(^3\)H]inositol was assayed at 50 \(\mu\)M substrate concentration for 30 min. Values represent means ± S.D. of four or five independent experiments (number in parenthesis) with three or four oocytes each, after subtracting the values for water-injected oocytes as control. Wild-type inositol uptake was 51.4 ± 11.7 pmol/30 min/oocyte. For uptake studies in *L. donovani* parasites (right), MIT wild type and mutants were subcloned into the *Leishmania* expression vector pX-H (containing neo) and transfected into promastigote cells. myo-[\(^3\)H] Inositol uptake is given as a percentage relative to wild-type activity (1526.7 ± 290.5 pmol/min/10\(^8\) cells) after subtraction of endogenous inositol uptake (cross-hatched area) of control cells transfected with the vector (pX-H) alone. Solid bars in both panels indicate mutants for which inositol transport was significantly different (p < 0.001) from MIT wild type, analyzed by paired sample t test.

An increase in the uptake of inositol in the D19E mutant showed a similar effect as the protonophores and inhibited MIT uptake by the alternative carboxylate form and thus retained the charge. These conservative mutations both showed a significant reduction on inositol uptake compared with MIT wild type, but inositol uptake was 11-fold higher in D19E and 2-fold higher in E121D than in the respective nonconservative mutations. The protonophores are weak organic solvents, thus mito-V and the D19E mutant 44–50% but stimulated inositol uptake (data not shown).

**Immunolocalization of MIT Mutants on Oocyte Plasma Membrane—**Immunofluorescence microscopy of oocyte cryosections showed that MIT mutants were expressed on the oocyte surface in similar quantity as MIT wild type (Fig. 4), confirming that these mutations affect transport function, inhibiting inositol uptake up to 98%, and do not prevent trafficking of the transporter to the plasma membrane. Water-injected oocytes served as control and did not show any plasma membrane staining (Fig. 4). MIT was expressed homogeneously over the entire oocyte plasma membrane of both animal and vegetal pole. The results shown in Fig. 4 were confirmed by examining four separate oocytes expressing each MIT mutant.

**Effect of Proton Gradient Uncouplers and pH on Inositol Uptake by MIT Mutants—**Subsequently, we investigated the effect of two protonophores and uncouplers on the various mutants. The proton gradient uncouplers FCCP and dinitrophenol inhibited inositol transport by 50–70% in the wild-type as well as in the control mutant D32N. The same protonophore sensitivity was found in the MIT mutants E95Q and E121Q, despite the reduced transport activity of the latter one (Fig. 5). Surprisingly, one mutant, D19N, completely lost all inhibition and instead was stimulated about 4-fold by either protonophore (statistical significance: p < 0.002, paired sample t test). Subsequently, we further investigated the pharmacology and pH dependence of this interesting Asp19 mutant (Table III). Phenol did not show the stimulatory effect on D19N and served as control for the protonophore effect of dinitrophenol, separating from its effects as an organic solvent. Monensin had no effect on inositol uptake in any of the Asp19 mutants or MIT wild type, and as a Na\(^+\) ionophore it served as control for the H\(^+\) specificity of the D19N effect (Table III, top). Finally, cyanide showed a similar effect as the protonophores and inhibited MIT wild type and the D19E mutant 44–50% but stimulated inositol uptake about 2-fold in the D19N mutant (p < 0.02). This latter reagent was used to indirectly reduce the proton gradient by affecting H\(^+\)-ATPase activity, due to the inhibition of cyto-
chrome activity and reduction of ATP levels in the cells.

A reversal of pH dependence of inositol uptake in the D19N mutant compared with MIT wild type and D19E (Table III, bottom) further supported the results obtained by the proton gradient uncouplers. Inositol uptake in the D19N mutant was stimulated about 1.7-fold when the extracellular proton concentration was reduced from pH 7.5 to pH 8.5, whereas MIT wild type was inhibited about 60% at pH 8.5. Conversely, an increased extracellular proton concentration at pH 6.5 stimulated inositol uptake in MIT wild type about 3.5-fold compared with pH 7.5, while D19N was inhibited about 10% (Table III, bottom). The cumulative data in Table III show that the conservative mutant D19E has the same inhibition profile as MIT wild type and, together with the other controls, suggest that the stimulatory effect of protonophores or reduced extracellular proton concentration upon D19N results from the removal of the charge of the carboxylate and a reduction of the cell’s proton gradient. These results underscore the probable importance of aspartate 19 in proton symport, although we do not currently understand how this stimulation occurs. One possibility is that alteration of the transporter’s protonation may lead to conformational changes in the D19N mutant and the observed stimulatory effect on inositol transport.

**DISCUSSION**

Acidic amino acid residues have been shown to be important in translocation of protons across membranes in bacteriorhodopsin, where Asp\(^{105}\) and Asp\(^{106}\) have been postulated to play...
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**Table III**
Pharmacology and pH dependence of inositol uptake in MIT wild type and D19 mutants

*Xenopus* oocytes were injected with *in vitro* transcribed MIT cRNA and assayed for myo-inositol uptake in the presence of (top) various inhibitors or 0.5% ethanol as control, or (bottom) various pH values of the uptake medium. Water-injected oocytes served as control to determine MIT-specific inositol uptake. Data were obtained at 50 μM inositol concentration from three or six independent experiments (mean ± S.D.; 100% inositol uptake equal to 51.4, 0.93, or 10.5 pmol/30 min/oocyte for MIT wild type, D19N, or D19E mutant, respectively. Statistical significance compared with wild-type: *, *p < 0.10; **, *p < 0.02; ***, *p < 0.001 (paired sample t test).

| Inhibitor       | Wild-type | D19N | D19E |
|-----------------|-----------|------|------|
| Control, 1% ethanol (6) | 100      | 100  | 100  |
| FCCP, 10 μM (6)   | 41.2 ± 3.5| 41.2 ± 3.5| 41.2 ± 3.5|
| DNP, 1.5 mM (3)   | 34.3 ± 15.4| 34.3 ± 15.4| 34.3 ± 15.4|
| Phenol, 1.5 mM (3) | 104.8 ± 15.6| 104.8 ± 15.6| 104.8 ± 15.6|
| Monensin, 2 μM (3) | 98.7 ± 6.8 | 98.7 ± 6.8 | 98.7 ± 6.8 |
| NaCN, 1 mM (3)    | 50.4 ± 15.3| 50.4 ± 15.3| 50.4 ± 15.3|
| pH of uptake medium | 345.9 ± 52.6| 345.9 ± 52.6| 345.9 ± 52.6|
| pH 6.5 (3)       | 89.6 ± 4.3**| 89.6 ± 4.3**| 89.6 ± 4.3**|
| pH 7.5, control (3) | 100      | 100  | 100  |
| pH 8.5 (3)       | 174.5 ± 35.1**| 174.5 ± 35.1**| 174.5 ± 35.1**|
| % (pH/control)   | 122.4 ± 18.1| 122.4 ± 18.1| 122.4 ± 18.1|
| % (inhibitor/control) | 100      | 100  | 100  |

Direct roles in transfer of protons from inside to outside of the membrane (17). In the *lac* permease, Asp³⁵⁵ and three positively charged residues, His³³², Arg³⁵², and Lys³¹⁴, have been proposed as important residues for transport (16). Furthermore, in the *E. coli* melibiose permease, the acidic transmembrane residues Asp³¹, Asp³⁵, Asp³⁵³, and Asp³⁵⁴ have been implicated in interaction with both the H⁺ and Na⁺ cosubstrates (19). Remarkably, alteration of these residues changes the counterion specificity of this transporter. The apparently central role of charged transmembrane residues in transporter function encouraged us to identify and alter, by mutagenesis, such residues in MIT. Indeed, we have found that alteration of two of the three charged transmembrane residues almost eliminates transport activity. Although we do not know the specific function of Asp⁹⁵ or Glu¹²¹ in MIT, these amino acids are likely candidates to be directly involved in transport mechanism. Conversely, alteration of Glu¹²¹ to Gln does not affect transport function, indicating that alteration of any charged transmembrane residue is not sufficient to eliminate transport function.

Subsequently, we compared the conservation of the three acidic transmembrane residues in MIT with five inositol and sugar/proton symporters from bacteria and yeast (Fig. 6), all sharing an active transport mechanism. Acidic amino sequence alignment of the corresponding transmembrane domains 1, 3, and 4 was analyzed and quantitated through the variability profile as the number of different amino acids occurring at each locus (33). Asp³⁵ and Glu¹²¹ of MIT were 100% conserved in all six proton symporters, whereas Glu⁹⁵ was not conserved, with three different amino acids found at that locus in the six transporters (Fig. 6). Conservation of amino acids is a sign of their potential significance with regard to protein function, and these findings match our mutagenesis studies and underscore the importance of Asp³⁵ and Glu¹²¹ for MIT function. While the MIT Asp³⁵ position is an aspartate in these six proton symporters, it is an asparagine in the homologous facilitated diffusion sugar transporter from lysosomes (34). The possible importance of Asp³⁵ for proton binding is underscored by the pharmacology of MIT wild type and Asp³⁵ mutants (Table III). Active proton-coupled inositol transport is found for a carboxylate side chain at residue 19 (Asp³⁵ or Glu³⁵), as suggested by inhibition with protonophores or cycloheximide. The mutant with the uncharged amide Asn³⁵, in contrast, is not inhibited by proton uncouplers. Currently, we cannot fully explain the stimulatory effect of a reduced proton gradient upon the D19N mutant, other than a possible allosteric effect that may be caused by changes in the protonation of the trans-

![Image](image-url)
Comparison of the three transmembrane domains 1, 3, and 4 in MIT, ITR1, ITR2, AraE, GalP, and YxIE (Fig. 6) revealed a significantly higher degree of conservation in helices 1 and 4 with 66.2 and 62.0% amino acid identity, respectively, compared with helix 3 with 44.9% identity. In helix 1, identity among the six proton symporters increases to 84.9% for the carboxyl-terminal half of the transmembrane domain (MIT G13-I23), which contains residue Asp19. Remarkable is a peripheral orientation along a conserved and functionally important stretch of the transmembrane segment, potentially part of the substrate permeation pathway.

The Leishmania MIT is a member of the large sugar transport superfamily, ranging from bacteria to plants and mammals, and this protozoan transporter serves as a model for active transporters in early eukaryotes. The bacterial disaccharide/cation symporters, lactose permease and melibiose permease, are usually grouped in a separate family, based on structural and functional data (35, 10, 11). For lac permease it was demonstrated that transmembrane domains 6–12 (designated C6) are sufficient to catalyze facilitative transport (18), and four charged transmembrane residues essential for transport function are located in C6. This is distinct from MIT, where charged residues important for function are in the NH2-terminal half of the carrier. However, four aspartate residues in the NH2-terminal half of the melibiose transporter are thought to be important for counterion binding (36), similar to the results obtained with MIT.

Site-directed mutagenesis in the small number of transporters extensively studied has revealed that residues critical for transport activity are rather rare, and in the mammalian glucose transporter GLUT1 only two residues (Gln161 and Trp412) were found to greatly influence transport activity (37). These residues lie within transmembrane domains 5 and 11, respectively, and support the idea based on ligand binding and labeling studies that the C-terminal part of GLUT1 may form the aqueous pore for the substrate permeation pathway (11, 38). In MIT, however, transmembrane domains 1 and 4 appear to be important for substrate transport. The observation that either NH2-terminal or C-terminal half of the various transporters are implicated to align the substrate translocation pathway may be explained by the symmetrical arrangement of two six-helix bundles, derived from an internal gene duplication event in an ancestral six-transmembrane domain transporter (35, 11), where one of the two six-helix bundles retains the substrate translocation pathway in the modern 12-helix transporter. However, this hypothesis has been challenged recently by a photoaffinity labeling study of GLUT1 (39) showing evidence that not only the carboxyl-terminal half but also the amino-terminal half of GLUT1 helices participates in the putative substrate channel formation.

In this study, we have shown the importance of two transmembrane carboxylate residues for transport function in a protozoan proton symporter, similar to results observed with active transporters from bacteria. These residues may play a direct role in the transport of protons, although this conjecture remains to be proven. For most transporters, the location of the “permeation pathway” is still largely unknown. It is likely that Asp19 and Glu121 are on this permeation pathway in MIT. The results presented here suggest experiments to test the possibility that Asp19 and Glu121 are on this transport pathway, including systematic chemical modification of residues on putative transmembrane helices 1 and 4, both in the presence and absence of myo-inositol (40, 41).

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