Four Conserved Cytoplasmic Sequence Motifs Are Important for Transport Function of the Leishmania Inositol/H\(^+\) Symporter*

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The protozoan Leishmania donovani has a myo-inositol/proton symporter (MIT) that is a member of a large sugar transporter superfamily. Active transport by MIT is driven by the proton electrochemical gradient across the parasite membrane, and MIT is a prototype for understanding the function of an active transporter in lower eukaryotes. MIT contains two duplicated 6- or 7-amino acid motifs within cytoplasmic loops, which are highly conserved among 50 members of the sugar transporter superfamily and are designated A\(_1\), A\(_2\) (V(D/E)(R/K)GR(R/K)), and B\(_1\), B\(_2\) (PESPR(4L), VPETKG). In particular, the three acidic residues within these motifs, Glu\(^{187}\)(B\(_1\)), Asp\(^{300}\)(A\(_2\)), and Glu\(^{429}\)(B\(_2\)) in MIT, are highly conserved with 96, 78, and 96% amino acid identity within the analyzed members of this transporter superfamily ranging from bacteria, archaea, and fungi to plants and the animal kingdom. 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 these three acidic residues in the B\(_1\), A\(_2\), and B\(_2\) motifs. Alteration to the uncharged amides greatly reduced MIT transport function to 23% (E187Q), 1.4% (D300N), and 3% (E429Q) of wild-type activity, respectively, by affecting \(V_{\text{max}}\) but not substrate affinity. Conservative mutations that retained the charge revealed a less pronounced effect on inositol transport with 39% (E187D), 16% (D300E) and 20% (E429D) remaining transport activity. Immunofluorescence microscopy of oocyte cryosections confirmed that MIT mutants were expressed on the oocyte surface in similar quantity to MIT wild type. The proton uncouplers carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone and dinitrophenol inhibited inositol transport by 50–70% in the wild type as well as in E187Q, D300N, and E429Q, despite their reduced transport activities, suggesting that transport in these mutants is still proton-coupled. Furthermore, temperature-dependent uptake studies showed an increased Arrhenius activation energy for the B\(_1\)-E187Q and the B\(_2\)-E429Q mutants, which supports the idea of an impaired transporter cycle in these mutants. We conclude that the conserved acidic residues Glu\(^{187}\), Asp\(^{300}\), and Glu\(^{429}\) are critical for transport function of MIT.

Parasitic protozoa of the genus Leishmania are important human pathogens (1) with more than 200 million people exposed to infection worldwide and an incidence of over 500,000 new cases annually of fatal visceral leishmaniasis (2). Transporters are of particular importance in these parasites to acquire nutrients from the host and offer attractive targets for rational drug design or vaccination. Myo-inositol plays a particularly important role in trypanosomatid protozoa like Leishmania as the precursor for various inositol phospholipids that are found in the great majority of surface molecules in these parasites. These include glycosylphosphatidylinositol-anchored surface glycoproteins like gp63 (3, 4) or abundant inositol-containing glycolipids (5, 6), several of which are involved in the invasion of macrophages in the mammalian host or the attachment to the midgut of the insect vector.

The myo-inositol/H\(^+\) symporter MIT\(^1\) from Leishmania donovani (7) is driven by a proton electrochemical gradient across the parasite membrane (8, 9). Active and concentrative uptake of nutrients is of specific importance in these parasites to compete successfully with the host for resources, and many transporters in trypanosomatid protozoa are thought to be proton symporters. Leishmania flagellates possess a particularly high membrane potential of about −115 mV (10) that serves as a powerful driving force for proton-coupled transport. Leishmania MIT is a well characterized proton symporter in trypanosomatid protozoa, and we have chosen MIT as a model for active transporters in early eukaryotes (11).

Primary and secondary structure amino acid analysis of MIT (7) revealed that it belongs to the large sugar transporter superfamily that contains both active and passive transport proteins for sugars and related small molecules (12, 13). This superfamily is ubiquitous in its distribution and contains 12-transmembrane domain transporters ranging from bacteria, cyanobacteria, and green algae to protozoa, fungi, and higher eukaryotes such as plants and mammals. For myo-inositol uptake in mammals, however, a Na\(^+\)/myo-inositol transporter SMIT has been identified (14) that is unrelated to the above sugar transporter superfamily but closely related to the intestinal Na\(^+\)/glucose transporter SGLT (15) and related sodium cotransporters like the vitamin transporter SVMT (16), which are members of the sodium/solute symporter superfamily (17). This difference between the mammalian and parasite myo-inositol transporters, together with the high abundance of inositol in flagellate surface molecules, suggests that MIT would make an attractive target for parasite-specific drug design.

Amino acid sequence alignment of MIT (7) with other transporters of the sugar transporter superfamily has revealed two duplicated sequence motifs A\(_1\), B\(_1\), and A\(_2\), B\(_2\) symmetrically

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The abbreviations used are: MIT, myo-inositol transporter from Leishmania; FCCP, carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone; BSA, bovine serum albumin; GLUT4, mammalian insulin-sensitive glucose transporter type 4; PBS, phosphate-buffered saline.
located on predicted cytoplasmic loops between membrane domains 2–3, 6–7, and membrane domains 8–9, 12-carboxyl terminus, respectively (Fig. 1). The symmetrical location of the motif RXRGR (A₁, A₂ in Ref. 18) was first noticed by Maiden et al. (19) in five bacterial sugar and citrate transporters and the human GLUT1 transporter, and an internal gene duplication event of an ancestral 6-transmembrane domain transporter was inferred from this observation (19). Subsequently, Szkutnicka et al. (18) recognized an additional, symmetrical PESPR and PETK sequence, designated B₁ and B₂, in the yeast galactose transporter GAL2 compared with four other sugar transporters from yeast and bacteria and the mammalian sugar transporters GLUT1, GLUT2, and GLUT3. In this study, we have compared the A₁, A₂, and B₁, B₂ motifs from Leishmania MIT and 48 representative other members of the sugar transporter superfamily to refine further and expand the conservation pattern in these motifs. The A and B motifs contain three or one positively charged residues each, but only one negatively charged restriction endonuclease site alteration adjacent to the aspartate residue are given in the single-letter code. Mutants are named as absence of any other sequence alterations. Mutants were sequenced for each of the three residues affecting silent restriction endonuclease site alteration. Oligonucleotide-directed, site-specific mutagenesis (23) was performed from MIT.pL2–5 plasmid as ND-96 buffer (22).

**TABLE I**

Mutations introduced into MIT

| MIT | Mutagenic primer | Nucleotide | Nucleotide change | Restriction site alteration |
|-----|------------------|------------|-------------------|-----------------------------|
| D32N | 5'-GAA GCC/GGA GTT/G+ G' GTT C/T CTG CAT CTG GAA-3' | 769 | G → A | New XmaI site |
| E187Q | 5'-G CCAG GGT/G+ G' GCT TGG CTG CAG CAG-3' | 1234, 1236 | G → C, G → A | BonII site lost |
| E187D | 5'-G CCAG GGT/G+ G' GCT TGG GTC CGC GAG-3' | 1236 | G → T | BonII site lost |
| D300N | 5'-ACC AAA GCC/GTT GA-GCG/GT GAA-3' | 1573 | G → A | SalI site lost |
| D300E | 5'-ACC AAA GCC/GTC GA-GCG/GT GAA-3' | 1575 | C → A | SalI site lost |
| E429Q | 5'-CTC CAG GCT/G+ AG GCC/TCT/CCT GCT CGC GGC3' | 1960, 1971 | G → C, G → A | BonII site lost |
| E429D | 5'-CTC CAG GCT/G+ AG GCC/TCT/CCT GCT GTC CGC GGC3' | 1962, 1971 | G → C, G → A | BonII site lost |

Italics indicate nucleotides replaced in mutants. The affected codon is underlined. Colons indicate beginning and end of the affected restriction site alteration.

**Materials and Methods**

**Parasite Culture and MIT Expression in Oocytes**—Promastigotes of the *L. donovani* DI-700 clone (WHO strain MHOM/SD/62/18; Ref. 20) were cultured at 27 °C in Dulbecco’s modified Eagle’s medium, adapted for *Leishmania* (DME-L medium; Ref. 21). Defolliculated stage V–VI *Xenopus laevis* oocytes were microinjected with 8–10 ng (40 nl) of capped MIT cRNA, which was transcribed in vitro as described previously (11). Injected oocytes were incubated for 3–5 days at 16 °C in ND-96 buffer (22). Site-directed Mutagenesis—Oligonucleotide-directed, site-specific in vitro mutagenesis (23) was performed from MIT.pL2–5 plasmid as described (11). Mutagenic oligonucleotides were designed to introduce a silent restriction endonuclease site alteration adjacent to the aspartate or glutamate mutation (Table), and mutant clones were identified by restriction enzyme mapping. The presence of the mutation was verified by DNA sequencing for all seven mutants (24). Additionally, the entire mutant gene was sequenced for each of the three residues affecting the specific nonconservative mutant (E187Q, D300N, and E429Q) to confirm the introduction of the desired mutation and the absence of any other sequence alterations. Mutants are named as wild-type residue, residue number, and mutant residue, in which the residues are given in the single-letter code.

**Plasmid Constructs and Transfection into Leishmania**—For expression of the MIT gene in oocytes, the plasmid MIT.pL2–5 was generated (Ref. 11; *Xenopus* expression vector pL2–5 kindly provided by Dr. Susan Amara, Vollum Institute). For MIT overexpression 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-Neo (kindly provided by Dr. Stephen Beverley, Washington University) to produce the MIT.pX-H plasmid (11). Transfection of *L. donovani* promastigotes was performed by electroporation using standard methods (25), and transfectants were selected in liquid medium containing 200 μg/ml of neomycin analog, G418 (Life Technologies, Inc.).

**Transport Assays—myo-[2-3H]Inositol** (specific activity of 21 Ci/mmol; NEN Life Science Products) was utilized for all transport assays in Leishmania and oocytes. Promastigotes from mid 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 of radiolabeled myo-inositol at 50 μM final concentration in PBS was measured at 25 °C within the linear uptake range between 10 and 120 s and terminated by spinning the cells in microcentrifuge tubes through an oil cushion of dibutyl phthalate (Sigma) followed by immediate snap-freezing of the tube in a dry ice/ethanol bath (26). Subsequently, the tip of the tube with the frozen cell pellet was clipped off into 250 μl of 1% SDS, mixed with 2 ml of Ecomove (ICN, Costa Mesa, CA), and analyzed by liquid scintillation counting. Linear regression analysis was used to determine the initial myo-inositol uptake rate from the linear uptake range for the various transfectants. Temperature-dependent myo-inositol uptake assays were performed at 20, 22, 24, 26, and 28 °C in a thermal cycler, and from the Arhenius plot the activation energy for inositol transport was determined.

Transport measurements in *Xenopus* oocytes were performed at room temperature for 30 min in 300 μl of radiolabeled myo-inositol (50 μM to 3 μM final concentration) in ND-96 buffer. Uptake was terminated by washing the oocytes 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 (11). Values of water-injected control oocytes were subtracted to determine MIT-specific inositol uptake. For inhibitor studies, the proton uncouplers FCCP or dinitrophenol (both applied from an ethanol stock solution) were preincubated for 10 min with the oocytes prior to 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 with two-tailed p values (27). For the substrate saturation kinetics, Kₘ and Vₘₐₓ values were determined by least squares fit of the data to the Michaelis-Menten equation, employing the Levenberg-Marquardt algorithm of KaleidaGraph program (Synergy Software) (22).

**Confocal Immunofluorescence Microscopy**—For MIT immunolocalization in oocyte crosssections, MIT-expressing oocytes (4 per wild type or mutant) were fixed in 3% 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, Torrance, CA) for 5–8 h at room temperature (28). Embedded samples were rapidly frozen in cryostat or in cryoslices mounted on cryo-tolysoine-coated slides, dried, and stored at −20 °C. Antibody against the 136-kDa carbohydrate terminus of MIT (MIT-COOH) as GST fusion protein was used for subsequent immunolocalization. Briefly, oocyte sections blocked with 5% BSA in PBS for 10 min were incubated with MIT-COOH rabbit antiserum (diluted 1:100 in 1% BSA/PBS) for 1 h and Texas Red-conjugated secondary antibody (Molecular Probes, Eugene, OR; diluted 1:500 in 1% BSA/PBS) for 30 min. A Leica confocal-laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a Leica 63× oil immersion lens was used to examine the oocyte sections (29). Prior to the embedding procedure, oocytes from the same microinjection were tested for mutant-specific inositol uptake, and oocytes from the same batch were used to produce the crosssections shown in Fig. 4 to control for variability between different oocyte batches and 8–10 μm were cut on a cryostat.
Conserved Cytoplasmic Motifs of Leishmania Inositol/H⁺ Symporter

Comparison of the conserved motifs A₁, A₂, B₁, and B₂ in the amino acid sequence of MIT with 50 members of the sugar transporter superfamily (aligned according to Fig. 2). The percent amino acid identity for each residue is given in parentheses below the general motif, and residues with more than 75% identity are underlined. For the acidic residues mutated in this study (boldface type), amino acid identity was 96% (E in B₂), 78% (D/E in A₂) and 96% (E in B₂), respectively. Φ, a hydrophobic residue; F, Y, W or G, A, V, L.

| MIT motif | Sugar transporter superfamily |
|-----------|-----------------------------|
| A₁ (loop 2) | AAFGRR |
| B₁ (loop 6) | PE₁⁸⁷SPRL |
| A₂ (loop 8) | VD₃⁰⁰RGGR |
| B₂ (COOH tail) | AVE₁⁴⁷TKG |

TABLE II

Conserved cytoplasmic sequence motifs in MIT and members of the sugar transporter superfamily for many members of this family conforms to the pattern (X+XX+++) for the A motifs and (XX(X)+XX) for the B motifs (Table II). The three acidic residues within these motifs, glutamate 187 (motif B₁), aspartate 300 (motif A₂), and glutamate 429 (motif B₂) in MIT, are highly conserved and show 96, 78, and 96 amino acid identity with the other members of this transporter superfamily. L. donovani MIT has four potential phosphorylation sites for cAMP-dependent protein kinase (I/R/XXX(S/T)) and four potential calcium/calmodulin-dependent protein kinase sites (S/T/X/I/R/K) within the entire amino acid sequence. In the 50 transporters analyzed, two of the cAMP-dependent protein kinase sites are immediately adjacent to the A₁ and B₂ motifs in 56 and 58% of the transporters, respectively, and one of the calcium/calmodulin-dependent protein kinase sites is located within the B₂ motif in 70% of the carriers (Fig. 2).

It is notable that these motifs are divergent in 9 glucose transporters from Leishmania and trypanosomes but not in the plasmoidal glucose transporter or Leishmania myo-inositol transporters. Despite the general divergence of these motifs in trypanosomatid glucose transporters, the negative residue in both B motifs is nonetheless highly conserved (89% B₁ and 100% B₂) among family members. Together with the charge distribution, this conservation further prompted us to test the significance of the three acidic residues for MIT transport function by site-directed mutagenesis and functional expression in Xenopus oocytes and overexpression in Leishmania.

Inositol Uptake in MIT Mutants—Conversion of any of the three acidic residues Glu¹⁸⁷, Asp³⁰⁰, or Glu⁴²⁹ to the uncharged amide resulted in a dramatic reduction of inositol transport to 23, 1.4, and 2.8% of MIT wild-type activity, respectively, when the mutants were expressed in Xenopus oocytes (Table III). A control mutant, D₃₂N, in the first extracellular loop did not affect inositol uptake significantly, as reported previously (11).

For all oocyte assays, water-injected control oocytes from the same batch were assayed in parallel to determine MIT-specific inositol uptake. Background in these control oocytes was so low compared with MIT-injected oocytes that even the relatively low D₃₀₀N-specific and E₄₂⁹Q-specific inositol uptake was significantly different from uptake in water-injected oocytes (p < 0.005 for both E₃₀₀N and E₄₂⁹Q, paired sample t test). Subsequent transfection and overexpression of MIT in Leishmania flagellates confirmed the significance of Glu¹⁸⁷, Asp³⁰⁰, and Glu⁴²⁹ for MIT transport function with a reduction of transport by 98–99% compared with MIT wild-type overexpressors (Table III). MIT overexpressors showed 21-fold higher inositol uptake over endogenous uptake by flagellates trans-

RESULTS

Four Conserved Cytoplasmic Sequence Motifs—In order to identify functionally important sequence motifs in MIT, we have compared the MIT amino acid sequence with 48 representative members of the sugar transporter superfamily that represent each kingdom of living organisms, including bacteria, archaea, fungi, plants and animals (Fig. 2). MIT contains two duplicated 6- or 7-amino acid motifs within cytoplasmic loops that are highly conserved between the 50 transporter proteins analyzed, designated A₁ (AAFGRR), B₁ (PE₁⁸⁷SPRL), A₂ (VD₃⁰⁰RGGR), and B₂ (AVE₁⁴⁷TKG) in MIT, respectively (Fig. 1 and Table II). These motifs are located immediately adjacent to the cytoplasmic side of transmembrane helices 2, 6, 8, and 12. In the sugar transporter superfamily, these motifs can be summarized as A₁, A₂ (VD(E/D)(R/K)/ED(R/K)), and B₁, B₂ (VPETRG), with F indicating an aromatic amino acid. The charge distribution

* A. Seyfang, unpublished data.

FIG. 1. Secondary structure model of L. donovani MIT. Boxed areas indicate the 12 predicted hydrophobic transmembrane domains. Single-letter code is used to denote the conserved amino acids of the four cytoplasmic sequence motifs A₁, B₁, and A₂, B₂ (shaded). The position of acidic amino acids altered by site-directed mutagenesis is numbered. Amino acids D₁⁸⁷, D₃₀₀, and D₄²⁹ (in italics) are important for transport function of MIT as revealed from the functional expression of the mutants in this study.
**Conserved Cytoplasmic Motifs of Leishmania Inositol/H\(^+\) Symporter**

**Fig. 2.** Alignment of the four conserved cytoplasmic motifs A\(_1\), B\(_1\), A\(_2\), B\(_2\) of 50 members of the sugar transporter superfamily. *L. donovani* MIT and *L. mexicana* MIT were aligned with members of the transporter superfamily representing all five kingdoms eu-bacteria, archaea, fungi, plants, and animals (transporter designation as given under "Materials and Methods"). Adjacent transmembrane domains (TM) are overlaid on top of the columns. Amino acids shown with a black background fit the consensus shown at the bottom with "*+" indicating an Arg or Lys, and "*Φ" indicating an aromatic residue Phe, Lys, or Trp. Potential cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinase (PKC) phosphorylation sites are underlined with a dot marking the Ser/Thr residue for phosphorylation.

**Table III**

**Myo-Inositol uptake in MIT mutants expressed in *X. laevis* oocytes or overexpressed in *Leishmania* parasites**

For uptake studies in oocytes, *in vitro* transcribed cRNA was micro-injected, and uptake of myo-[\(^3\)H]inositol was assayed at 50 \(\mu\)M substrate concentration for 30 min. Values represent means + S.D. of three to five independent experiments (number in parentheses) with three or four oocytes each, after subtracting the values for water-injected control oocytes. Wild-type inositol uptake was 51.4 ± 11.7 pmol/30 min per oocyte. For uptake studies in *L. donovani* parasites, MIT wild-type and mutants were subcloned into the *Leishmania* expression vector pX-H (containing neo\(^r\)) and transfected into promastigote cells, *myo-[\(^3\)H]Ino*.

Inositol uptake is given in percent relative to wild-type activity (1526.7 ± 290.5 pmol/min per 10^8 cells) after subtraction of endogenous inositol uptake of control cells transfected with the vector alone.

| Inositol uptake | Xenopus oocytes | n | Leishmania transfectants | n |
|-----------------|----------------|---|--------------------------|---|
| % (mutant/wild type) | Wild type | 100 ± 23.3 | (5) | 100 ± 19.0 | (5) |
|                 | D32N | 115.3 ± 26.9 | (4) | 2.0 ± 0.8 | (4) |
|                 | E187Q | 22.5 ± 6.0 | (4) | 2.4 ± 0.8 | (4) |
|                 | E187D | 35.7 ± 3.2 | (3) | 1.6 ± 0.5 | (5) |
|                 | D300N | 12.6 ± 3.1 | (3) | 1.3 ± 1.0 | (4) |
|                 | E429Q | 28.2 ± 1.1 | (5) | 20.0 ± 3.7 | (3) |
|                 | E429D | 19.0 ± 4.3 | (4) | 15.6 ± 1.5 | (4) |

* Data were analyzed by paired sample t test, and mutants for which inositol transport was significantly different (\(p < 0.001\)) than MIT wild-type are indicated.

Conservative mutations of the three acidic residues to the alternative carboxylate form that retained the charge resulted in a less strong reduction of MIT transport between 16 and 39% of wild-type activity when compared with the nonconservative mutations (Table III). MIT transport was 1.7- (E187D), 12- (D300E), and 7-fold (E429Q) higher when the amino acid side chain retained the negative charge, and residues Asp\(^{300}\) and Glu\(^{121}\) in the carboxyl-terminal half of the transporter appeared to be particularly sensitive to removal of the charge (Table III).

Michaelis-Menten analysis of substrate saturation kinetics revealed that reduced inositol uptake in the Glu\(^{121}\), Asp\(^{300}\), and Glu\(^{129}\) mutants was due to a reduction of the \(V_{max}\) values by 85–97% compared with MIT wild-type activity (Fig. 3). \(V_{max}\) values dropped from 465 ± 152 pmol in wild-type cells and 556 ± 136 pmol (D32N) to 79 ± 4.3 pmol (E187Q), 15 ± 1.6 pmol (D300N), and 19 ± 1.5 pmol (E429Q) per 30 min and oocyte. In comparison with these dramatic reductions of \(V_{max}\) values, no significant changes of substrate affinity were found with \(K_m\) values of 209 ± 41 (E187Q), 966 ± 241 (D300N) and 542 ± 121 \(\mu\)M (E429Q) compared with 640 ± 144 \(\mu\)M in wild-type cell and 554 ± 121 \(\mu\)M for the D32N control.

**Immunolocalization of MIT Mutants—Confocal immunofluorescence microscopy of oocyte cryosections was used to investigate the cellular localization of MIT mutants. MIT-expressing Xenopus oocytes showed correct trafficking of MIT protein to the oocyte surface in similar quantity as MIT wild type (Fig. 4), confirming that these mutations affect transport function and do not prevent trafficking of the transporter to the plasma membrane. Water-injected control oocytes did not show any oocyte surface staining (Fig. 4). Furthermore, MIT protein was expressed homogeneously over the entire oocyte surface of both flagellates under drug selection (11).**

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animal and vegetal pole. Four separate oocytes expressing each MIT mutant were analyzed and revealed identical results to the ones shown in Fig. 4.

**Effect of Proton Uncouplers**—The proton gradient uncouplers FCCP and dinitrophenol inhibited inositol uptake by 50–70% in MIT wild type as well as in the control mutant D32N in the oocyte expression system, consistent with myo-inositol/proton symport of MIT (Table IV). The same protonophore sensitivity was found in the MIT mutants E187Q, D300N, and E429Q, despite their reduced transport activity (Table IV), suggesting that inositol transport in these mutants is still proton-coupled.

**Arrhenius Activation Energy**—Temperature-dependent inositol uptake between 20 and 28 °C was measured within the linear uptake range, and the Arrhenius activation energy was determined from the negative slope of an Arrhenius plot (Fig. 5). Interestingly, the activation energy of inositol transport was significantly increased for the two B-motif mutants B1-E187Q and B2-E429Q with 86.8 and 10.3 and 80.6 and 4.5 kJ per mol, respectively, compared with 64.2 and 65.5 ± 5.8 kJ per mol for MIT wild type and A2-D300N (Fig. 5, inset). This indicates that the rate-limiting step in the transporter cycle is slowed down for the B-motif mutants.

**Secondary Structure Analysis**—An analysis of secondary structure of MIT by the Chou-Fasman and the Robson-Garnier algorithms (MacVector 5.0 sequence analysis software) predicted a β-turn structure for all four cytoplasmic motifs analyzed in this study. This structure is supported by a residue of high β-turn propensity in the center of the motif (Gly in the A motifs and Ser/Thr in the B motifs) (Table II). Furthermore, the flexibility profile predicted a local flexibility maximum within all four motifs (MIT: first Arg in A1, Gly in A2, Ser in B1, and Thr-Lys in B2), compared with the neighboring 10 amino acids on both sides of the motifs. Predicted flexibility dropped rapidly around these maxima within 3–10 residues by 21–66% of the flexibility window found for *L. donovani* MIT. These flexibility profiles further support the variable loop in a β-turn structure.

**Fig. 3.** Concentration-dependent inositol uptake in MIT mutants. A, substrate saturation kinetics were determined in MIT-expressing oocytes for a concentration range of 50 μM to 3 mM myo-inositol (mean ± S.D. of 3 or 4 independent experiments). For each data point, the nonspecific inositol uptake by control water-injected oocytes was subtracted as described in Table III. B, enlargement for the E187Q, D300N, and E429Q mutants with reduced inositol transport activity revealed Michaelis-Menten-like saturable kinetics for all mutants.

**FIG. 4.** Confocal immunofluorescence micrographs of oocytes expressing MIT mutants. Oocytes were injected with cRNA or water as control and assayed for inositol uptake at 20, 24, and 28 °C. Myo-inositol uptake in the presence of protonophore FCCP or dinitrophenol, or 1% ethanol as control. Water-injected oocytes with identical treatment served as control to determine MIT-specific inositol uptake. Inhibition data are expressed relative to the control for each mutant independently (mean ± S.D. of two to six independent experiments with 3 or 4 oocytes each).

**TABLE IV**

| Inositol uptake       | Control | FCCP       | Dinitrophenol |
|-----------------------|---------|------------|---------------|
| % (inhibitor/control) |         |            |               |
| Wild type             | 100     | 45.7 ± 15.6| 34.3 ± 15.5   |
| D32N                  | 100     | 37.5 ± 0.7 | 29.0 ± 4.2    |
| E187Q                 | 100     | 28.5 ± 0.7 | 16.5 ± 0.7    |
| D300N                 | 100     | 23.4 ± 14.2| 10.5 ± 3.6    |
| E429Q                 | 100     | 41.9 ± 14.8| 27.9 ± 10.1   |

**DISCUSSION**

MIT is a member of the large and diverse sugar transporter superfamily (12, 13) which belongs to the major facilitator superfamily (34), also called the uniporter/symporter/antiporter superfamily (35). Many structure-function analyses of such transporters have focused on the location and analysis of transmembrane spanning domains to identify the substrate permeation pathway and substrate-binding sites in these permeases. Relatively little is known about the function of other regions outside the cell membrane in transporters, such as connecting loops or amino- and carboxyl-terminal tails. In this study we have analyzed and investigated four conserved and
functionally important motifs in hydrophilic cytoplasmic domains of the protozoan model proton symporter, *Leishmania* MIT.

Conservation of the two pairs of A and B motifs and their identical position within 50 transporters from a diverse transporter superfamily from bacteria to humans, together with chemically very diverse substrates for these permeases, from mono- and disaccharides to acidic Krebs cycle intermediates and antibiotics, suggest that these motifs are not directly involved in substrate recognition and specificity but that they are instead of particular functional importance in this class of transporters.

A conserved motif GXXXR/K/DGXXGXR/R(K/R) within transmembrane domain 2 and loop 2 has been studied in detail in *Escherichia coli* in the lactose permease (LacY) (36) and the tetracycline/H⁺ antiporter (TetB on Tn10) (37). This sequence contains the A₁ motif described here with an extension into membrane domain 2. Site-directed mutations in both transporters revealed that the functionally important residues are the first Gly of this motif in membrane domain 2 and the acidic residue of the A₁ motif (36, 37). For the tetracycline transporter the penultimate positively charged residue (Arg²⁶⁰) was also essential for transport function (37). Based on structural and functional data, the *E. coli* lactose permease is usually grouped in the oligosaccharide/proton symporter family (34), separate from the sugar transporter superfamily (19, 12, 13). Both LacY and TetB also have an A₂-like motif after membrane domain 8 which, however, shows a different charge distribution than A₁ or the A₂ motifs of other superfamily members (Table I), and there was no functionally essential residue in the A₂-like motif of the tetracycline/H⁺ antiporter (38). This is in striking contrast to the functional importance of Asp⁶¹⁰ in the A₂ motif of the MIT symporter which resembled more the conserved A₂ motif of most of the other members of this family (Fig. 2 and Table I) including the mammalian glucose transporters.

In the mammalian GLUT4 glucose transporter the importance of some of the charged residues of the A₁ and A₂ motif was recently investigated by site-directed mutagenesis followed by functional reconstitution in proteoliposomes and revealed the importance of the acidic residue (Glu³²⁹) in the GLUT4 A₂ motif (39), which is in agreement with our findings for *L. donovani* MIT. None of these studies in bacterial or mammalian transporters investigated the highly conserved B₁ or B₂ motifs that we analyzed in the present study, and the single acidic residue, Glu¹⁸⁷ or Glu⁴²⁹, in either of the MIT B-motifs appears to be critical for myo-inositol transport (Table III and Fig. 3) but not for proton coupling (Table IV).

Little is known about the function of the conserved amino acid motifs in other transporters. The duplicated RXGR motifs in A₁ and A₂ are predicted to form a β-turn between helices 2–3 and 8–9, respectively, and it was speculated that the multiple positively charged side chains may interact with neighboring phospholipid head groups (19). Furthermore, facilitation of conformational changes between the amino- and carboxyl-termini of the transporter by the A₁ and A₂ motifs was proposed recently from analysis of suppressor mutations of the lactose permease for parental strains that had transport-defective mutations in the A₁ (40) or A₂ motif (41). In addition, inhibitor binding and photolabeling studies of GLUT4 suggested that A₁-Arg⁹² or A₂-Arg³³³,Arg³³⁴ are required for substrate-induced conformational change of the carrier, whereas A₂-Glu³²⁹ mutations arrested the transporter in an inward-facing conformation (39). The increased activation energy for the two B-motif mutants in this study (Fig. 5) showed that the rate-limiting step for inositol transport in these mutants is slowed. Hence, our data for MIT support the idea that the impaired transporter cycle in the B-motif mutants is due to an impaired ability to undergo the conformational change. Furthermore, it is possible that the conserved A₁, A₂, B₁, and B₂ motifs of MIT could also be involved in interactions with the membrane or with other hydrophilic regions of the transporter. Experiments to test the role of these sequences in transporter function could include disulfide-scanning mutagenesis (42, 43) to introduce targeted disulfide cross-links between two respective cytoplasmic loops. Analysis of interactions between these domains may thus help to illuminate better the structural mechanisms that underlie the functional importance of the four conserved cytoplasmic motifs in this class of transporters.

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Conserved Cytoplasmic Motifs of Leishmania Inositol/H\textsuperscript{+} Symporter

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Four Conserved Cytoplasmic Sequence Motifs Are Important for Transport Function of the *Leishmania* Inositol/H\(^+\) Symporter

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