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Structure–function analysis of the highly conserved charged residues of the membrane protein FT1, the main folic acid transporter of the protozoan parasite *Leishmania*

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ABSTRACT

The main plasma membrane folate transporter FT1 of *Leishmania* belongs to the novel FBT family which is part of the major facilitator superfamily. We have investigated the role of the 10 most conserved charged amino acids of FBTs by site directed mutagenesis. The functions of the mutated proteins were tested for their capacity to transport FA, to sensitize methotrexate resistant cells to methotrexate, for protein production, and for protein localisation. Of the 10 conserved charged amino acids that were mutated to neutral amino acids, all had effects on FT1 transport activities. Only four of the 10 initial mutants (K116L, K133L, R497L, and D529V) retained between 15% and 50% of FT1 activity. The R497 residue was shown to be involved in substrate binding. When the charged conserved residues at position 124, 134, 179, 514, 537 and 565 were changed to neutral amino acids, this led to inactive proteins but the generation of new mutants D124E, R134K, D514E and D537E regained between 20% and 50% of wild-type FT1 activity suggesting that the charge is important for protein function. The mutated protein D179E had, under our standard experimental conditions, no activity, while E565D was completely inactive. The differential activity of the mutated proteins was due either to changes in the apparent K_m or V_{max}. Mutagenesis experiments have revealed that charged amino acids were essential for FT1 stability or activity and led to a plausible model for the transport of folic acid through FT1.

1. Introduction

Folic acid (FA) and related derivatives are conjugated pterins that, once reduced, are essential co-factors in one carbon transfer metabolism including the synthesis of thymidine, methionine and depending on the organisms of purines, serine, glycine and other molecules (reviewed in [1]). Some organisms are capable of de novo synthesis of FA (and of pterins) while others need to get these molecules from the environment. Membrane transport of FA is thus essential for several organisms and a diversity of membrane proteins are involved in the transport of FA derivatives. For example, the GPI-anchored membrane folate receptors mediate FA uptake by endocytosis and a number of facilitative organic anion carriers, including the reduced folate carrier, transport FA in mammals (reviewed in [2]). Recently, a proton coupled low pH folate transporter was isolated [3] and membrane transporters part of the ATP Binding Cassettes (ABC) family were shown to efflux FA derivatives (reviewed in [4]). Mammals [5] and plants [6] have mitochondrial carrier family proteins for the transport of FA into mitochondria. All these proteins are structurally different but have in common the transport of folate derivatives.

A further novel class of transporters, the Folate Biopterin Transporters (FBT) (www.tcdb.org/index.php) were recently described. They were first identified in the protozoan parasite *Leishmania* and were shown to transport biopterin [7,8] or FA [9,10]. Other members of the FBT family are likely to transport other substrates [11]. FBT homologues were found in the parasites responsible for sleeping sickness or Chagas diseases [11], malaria [12] or toxoplasmosis [13]. FBT homologues in cyanobacteria or in the plant Arabidopsis were also shown to transport FA and a number of derivatives [14]. The FA analogue methotrexate (MTX) is a widely used drug and the activity/mutations of some members of this diverse class of transporters can influence MTX accumulation and resistance to MTX. Due to the strategic importance of FA in one carbon transfer reaction a number of distinct membrane proteins have thus evolved in various organisms to transport FA derivatives.

The protozoan parasite *Leishmania* is distributed worldwide and is responsible for considerable morbidity and mortality [15,16]. Few drugs are available against this parasite but their FA metabolism is sufficiently different from their hosts that

Abbreviations: FA, folic acid; FBT, Folate Biopterin Transporter; MTX, methotrexate; NHS, *N*-hydroxysuccinimide; TMS, trans membrane segments.

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specific inhibitors of this pathway may eventually be found [1,17]. Indeed, several efforts are devoted to the generation of antifolates against *Leishmania* [18–21].

Leishmania is auxotroph for both pterins and folates and not surprisingly transport activities were described for these molecules in *Leishmania* [22,23]. The FA transport was decreased in MTX resistant cells [24–26]. The biopterin transporter BT1 was first isolated by functional cloning [7] and it was soon realised that this transporter is part of a family of proteins distantly related to the major facilitators. This family was named FBT (www.tcdb.org/index.php) and in addition to BT1, two more members were functionally characterized. FT5 corresponds to a high affinity/low capacity folate transporter [9] and FT1 to the main folate transporter of *Leishmania* [10]. The genome sequence of *Leishmania* has revealed 14 members of the FBT family with a varying degree of conservation but with several amino acids conserved not only between the *Leishmania* sequences but also in the FBT proteins found in other parasites, bacteria and plants. We used FT1 as the paradigm for studying FBT structure function relationship and have concentrated on conserved charged amino acids. The studies reported here are consistent with the importance of charged amino acids in the function of membrane proteins.

2. Material and methods

2.1. Parasites and culture

Leishmania tarentolae strain TarII MTX-1000.6 was described previously and has no measurable folic acid or methotrexate transport [26]. *L. tarentolae* promastigotes were transfected by electroporation as reported previously [27]. Growth inhibition was determined at various concentrations of MTX as described using $OD_{600\ nm}$ [28].

2.2. Western blot analysis

Total *Leishmania* proteins (30 μg) were run on 12% polyacrylamide gels and blotted onto nitrocellulose membranes as described [29]. The blots were blocked overnight in 5% skimmed milk in PBS. A monoclonal anti-α-tubulin antibody (Sigma, Oakville, ON, Canada) directed against a conserved amino-terminal peptide of the bovine α-tubulin or an antibody against the Green Fluorescent Protein (GFP) (Invitrogen, Burlington, ON, Canada) were diluted 1:3000 in PBS containing 0.1% Tween 20 (PBS–Tween) and incubated for 1 h with the blot. The blot was washed 3 × 5 min in PBS–Tween and incubated with horseradish peroxidase-conjugated sheep-anti mouse IgG for α-tubulin and sheep-anti rabbit IgG for GFP (Amersham Biosciences, Baie D'Urfé, QC, Canada) diluted 1:10,000 in PBS–Tween. Reactions were revealed with the ECL Plus chemiluminescent substrate (Amersham Biosciences, Baie D'Urfé, QC, Canada), and autoradiography.

2.3. Site directed mutagenesis

Mutagenesis was performed using the quick change site-directed mutagenesis kit (Stratagene, Mississauga, ON, Canada). Mutagenic primers were designed to incorporate the desired mutations in FT1. Mutations were inserted within the *FT1* open reading frame fused with GFP at its C-terminus. After sequencing of the whole open reading frame, mutated *FT1GFP* were subcloned into the *Leishmania* expression vector pSPαHYGα [30]. A total of 19 point mutations were generated and are listed in Table 1.

2.4. Confocal microscopy

Parasites were washed in PBS, and resuspended at a density of 10^7 cells/ml. Cells were immobilized in a 0.5% low melting agarose PBS buffer and mounted on microscope slides with coverslips. Samples were viewed with an Olympus FV300 confocal scanning laser system installed on an Olympus IX-70 inverted microscope with an argon laser. Visualization of the fluorophore was achieved using a 488 nm excitation filter and 510/530 nm emission filter. Samples were scanned for green fluorescence using a 100× objective (numerical aperture, 1.60). Images were obtained using the Olympus Fluoview 300 software.

Table 1
Activities of mutated versions of FT1.

Amino acid	Conservation[a] (%)	MTX susceptibility (μM)[b]	Protein production[c]	Folate uptake (pmol/10^8 cells/min)[d]
WT	–	–	–	0.22 ± 0.05
MTX1000.6	–	>1000	–	0.02 ± 0.01
FT1GFP-WT	–	<50	+	1.44 ± 0.17
-K116L	89	<50	+	0.63 ± 0.15
-D124V	100	>1000	+	0.03 ± 0.02
-D124E	100	420	+	0.19 ± 0.06
-K133L	57	<50	+	0.64 ± 0.13
-R134L	89	>1000	+	0.03 ± 0.02
-R134K	89	<50	+	0.59 ± 0.08
-D179V	89	>1000	+	0.05 ± 0.04
-D179E	89	350	+	0.03 ± 0.03
-E270L	55	<50	+	1.14 ± 0.17
-R497L	92	<50	+	0.65 ± 0.29
-D514V	78	>1000	–	0.01 ± 0.01
-D514E	78	<50	+	0.29 ± 0.08
-D514N	78	>1000	+	0.01 ± 0.01
-D529V	92	<50	+	0,25 ± 0,05
-D529E	92	<50	+	0.45 ± 0.02
-D537V	81	>1000	+	0.04 ± 0.02
-D537E	81	<50	+	0.86 ± 0.01
-E565L	100	>1000	+	0.02 ± 0.01
-E565D	100	>1000	+	0.02 ± 0.01

[a] % Conservation at the same position among the 37 proteins of the alignment.
[b] MTX susceptibility of *L. tarentolae* MTX 1000.6 strain expressing varying versions of FT1. Average of triplicate measurements.
[c] Detection of the production of FT1GFP proteins by Western blot and their localisation within the plasma membrane.
[d] Folate transport after 10 min. Average of triplicate measurements.