Hexose uptake in *Trypanosoma cruzi*: structure–activity relationship between substrate and transporter

Emmanuel TETAUD*, Sandrine CHABAS, Christiane GIROUD, Michael P. BARRETT* and Théo BALTZ†

Laboratoire Biologie Moléculaire et Immunologie de Protozoaires Parasites, Université Bordeaux II, URA 1637, Centre National de la Recherche Scientifique, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France

The gene encoding a hexose transporter, TcrHT1, from *Trypanosoma cruzi* has been functionally expressed in mammalian Chinese hamster ovary cells. Kinetic parameters of the heterologously expressed protein are very similar to those of the transporter identified in *T. cruzi* epimastigotes, confirming that TcrHT1 is the major transporter functioning in these parasites. A detailed analysis of substrate recognition using analogues of D-glucose substituted at each carbon position has been performed. The glucose transporter of *T. cruzi* does not recognize C-3 or C-6 analogues of D-glucose, whereas these analogues were recognized by the glucose transporter of bloodstream-form *T. brucei*. As for other kinetoplastid transporters, but in stark contrast to the mammalian GLUT family, TcrHT1 can also transport D-fructose, with relatively high affinity (K_m = 0.682 ± 0.003 mM). Amino acid side-chain-modifying reagents were also used to identify residues of the transporter present at the substrate-binding site. While specific modifiers of cysteine, histidine and arginine all inhibited catalytic activity, protection using substrate was only observed using the arginine-specific reagent, phenylglyoxal. Reagents which modify lysine residues had no effect on transport.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi* causes the potentially debilitating Chagas' disease to which millions of people in Central and South America are constantly exposed. Currently there are no satisfactory drugs for use against this malady and no vaccines exist. Nifurtimox, the drug of choice, suffers from the drawback of high cost and unacceptable side effects including allergies and damage to the central nervous system. The mode of action of trypanocidal reagents is unknown, and currently used compounds were derived empirically. To increase the chances of success, the development of new drugs should be aimed at those steps in a metabolic pathway which are either absent or differ from analogous steps in the host. Glucose carrier molecules present in the various members of the family *Trypanosomatidae* meet this requirement. For example, unlike the mammalian glucose transporters, the carrier molecule of another trypanosome (*T. brucei*) demonstrates a relatively high affinity for D-fructose [1,2]. Moreover, in providing a gateway between the cytosol and host environment, any transporter potentially may be exploited in the uptake of drugs.

The transport of glucose in trypanosomatids has been extensively studied at both the biochemical and genetic levels [3–20]. A gene encoding a protein of the glucose transporter superfamily has been identified in *T. cruzi* and it has been shown to encode a hexose transporter by expression in *Xenopus* oocytes [21]. Uptake of drugs into a parasite is a prerequisite for any intracellular toxic activity. The hexose transport system currently represents the best understood, and most easily studied, portal between *T. cruzi* and its environment. An understanding of atomic components of the transporter's physiological substrate involved in recognition is necessary if molecules with potentially harmful function are to be developed for delivery to the parasite via this route. In addition to confirming that the TcrHT gene encodes the protein responsible for glucose uptake in *T. cruzi*, we report here the hydrogen-bonding requirements between substrate and the *T. cruzi* transporter. Moreover the development of a functional expression system for the *T. cruzi* hexose transporter gene in Chinese hamster ovary (CHO) cells has allowed the investigation of amino acid residues involved in recognition of the substrate.

MATERIALS AND METHODS

Trypanosomes

T. cruzi strain C. L. epimastigote forms were cultured and prepared as described [22].

Chemicals and reagents

All chemicals were of the highest quality available from Sigma. 2-Deoxy-D-[1-^{3}H]glucose (2-DOG), D-[U-^{14}C]glucose and D-[U-^{14}C]fructose were from Amersham.

Preparation of trypanosomes for transport studies

T. cruzi epimastigotes were centrifuged at 2000 ***g*** for 10 min at 20 °C. The resulting pellet was washed three times in PBS

Abbreviations used: CHO, Chinese hamster ovary; 2-DOG, 2-deoxy-D-glucose; NEM, *N*-ethylmaleimide; PG, phenylglyoxal; DEP, diethylpyrocarbonate; NBS, *N*-bromosuccinimide; EAI, ethylacetamidate; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

* Present address: Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K.
† To whom correspondence should be addressed.

[1.8 mM KH_2PO_4/5 mM K_2HPO_4/0.9% (w/v) NaCl, pH 7.4] and finally resuspended in PBS. The centrifugation-through-oil method was used to determine transport of D-glucose or D-fructose at 22 °C as described [9]. The K_i values for analogues were calculated by the method of Eisenthal et al. [1] at a concentration of 10 μM D-glucose or D-fructose, using the equation $v_o/v = 1 + I/K_i$, where v_o and v are the uninhibited and inhibited rates respectively, and I is the inhibitor concentration. The K_i value, was determined from the plot v_o/v versus competitor concentration. The protein concentration of cells in the final suspension was determined by the method of Bradford [23], using BSA as standard protein.

Expression of the TcrHT1 gene in CHO cells

The TcrHT1 gene, including the sequence implicated in the translation initiation of eukaryote mRNA [24], was cloned directionally into the expression vector pCDNA3 (from Invitrogen) to yield plasmid pCHO-TcrHT1 for transfection into CHO-K1 cells. CHO-K1 cells were maintained in Iscove's medium containing 10% (v/v) fetal calf serum. Cells were transfected by the calcium phosphate method [25] and selected by their resistance to the neomycin analogue, G-418 (Gibco), at 700 μg/ml. Cells expressing TcrHT1 were identified by assaying transfected lines for their ability to transport 2-DOG in the presence or absence of 10 μM cytochalasin B, and their ability to transport D-fructose in the presence or absence of 5 mM D-glucose.

Assay of 2-DOG uptake in CHO cells

Cells were grown to a density of 5×10^5 cells per well in 24-well plates. Prior to initiating uptake assays, cells were washed three times with PBS and incubated in 0.3 ml of PBS containing 10 μM cytochalasin B for 5 min to inhibit the endogenous transporter. Sugar uptake was initiated by the addition of 0.1 ml of PBS containing 2-DOG at a final concentration of 0.1 mM (1 μCi 2-deoxy[1-^{3}H]D-glucose) followed by incubation for 5 min at 22 °C. Uptake was terminated by three rapid washes with 0.3 ml per well of ice-cold PBS. Cells were solubilized with 0.2 ml of 2% SDS, and incorporated radioactivity was detected by liquid scintillation counting. The effect of inhibitors or competitors on 2-DOG transport was studied by incubating cells with the inhibitor or competitor for 5 min prior to the commencement of the transport assay. We have also tested uptake without pre-incubation of competitors or inhibitors, and no differences were observed.

Chemical modification

The effect of modifying reagents on D-glucose transport in *T. cruzi* epimastigote and 2-DOG transport in CHO cells were studied by incubating cells with the reagents for 5 min then removing unbound reagent by washing three times in PBS prior to the commencement of the transport assays which were carried out as reported above. The incubations with *N*-ethylmaleimide (NEM) and phenylglyoxal (PG) were performed in PBS (pH 7.4), those with diethylpyrocarbonate (DEP) and *N*-bromosuccinimide (NBS) in PBS (pH 6) and those with ethylacetamidate (EAI) and 2,4,6-trinitrobenzenesulphonic acid (TNBS) in PBS (pH 8). DEP was dissolved in ethanol.

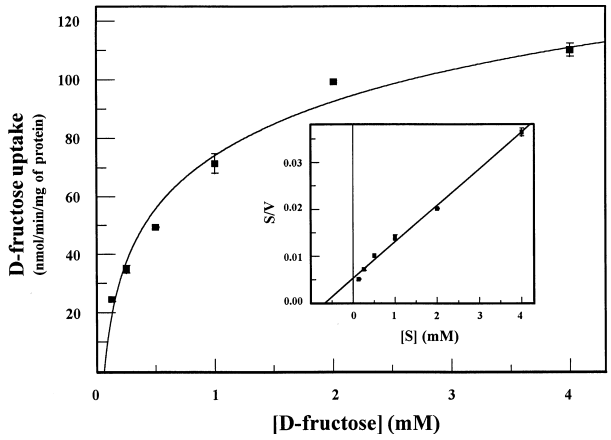

Figure 1 Kinetics of initial D-fructose uptake

The rate of D-fructose transport was measured over the concentration range 0.125–4 mM at 22 °C. Assays were conducted by determining the initial rate of transport (10 s time point), using an oil centrifugation method. Values are expressed as mean ± S.D. ($n = 4$). Inset: Hanes plot.

RESULTS

Kinetics of D-glucose and D-fructose uptake in *T. cruzi*

We have previously determined the kinetics and specificity of the glucose transporter in *T. cruzi* epimastigotes [21]. Zero-trans kinetic parameters, previously determined using double-reciprocal plots, were recalculated using Hanes' plots. Plotting the data via this route gave significantly altered kinetic parameters ($K_m = 0.312 \pm 0.01$ mM for 2-DOG as opposed to $K_m = 0.154 \pm 0.004$ mM). This discrepancy has almost certainly arisen due to linear regression of data transformed to a double-reciprocal plot, where the lowest substrate concentration has biased the slope of the line.

Kinetics of D-fructose uptake were also studied in *T. cruzi* epimastigotes. Uptake was linear for 2 min using 0.5 mM substrate. A plateau was not obtained, since D-fructose is metabolized (results not shown). Kinetics were measured using a 10 s time point, and a saturable process was observed (Figure 1). The plot of $[S]/V \times [S]$ was linear, implying that a single transport system is operative over the range studied (0.01–4 mM). The apparent K_m was 0.682 ± 0.003 mM and the V_{max} was 128.7 ± 2.4 nmol/min per mg of protein.

To determine whether D-fructose and D-glucose are transported via a common transporter, we compared the K_m values for these substrates with their K_i values. The K_m value for D-fructose is similar to the K_i value previously determined (Table 1), and D-glucose inhibited D-fructose transport with a K_i value of 0.074 ± 0.002 mM, which is very close to the K_m value observed for D-glucose transport (Table 1). The small difference between K_m and K_i values for D-fructose can be explained by the differences in technique for the determination of these values and the metabolism of D-fructose. K_i values for D-fructose against D-glucose and vice versa are similar to their respective K_m values, indicating that D-glucose and D-fructose probably share the same transporter, as seen for bloodstream and procyclic forms of *T. brucei* [2,26].

Specificity of hexose transport in *T. cruzi*

In order to further characterize the substrate specificity, D-glucose analogues, substituted at all six carbon positions, were used as

Table 1 Inhibition constants (K_i) for D-glucose analogues

The K_i values for *T. cruzi* were determined by inhibition of D-glucose uptake using a 10-s time point assay at 22 °C. Values are expressed as means ± S.D. ($n = 4$). Other values are from refs. *[1], †[21], ‡[7], and §[2]. **K_m not K_i.

Position substituted	Analogue	*T. cruzi* epimastigote K_i (mM)	*T. brucei* bloodstream form K_i (mM)*	*T. brucei* procyclic form K_i (mM)
	D-Glucose	0.059 ± 0.010†	0.90 ± 0.04	0.045‡
C-1	1-Fluoro-1-deoxy-D-glucose	1.39 ± 0.060	2.89 ± 0.63	0.750§
C-2	D-Mannose	0.025 ± 0.001†	0.67 ± 0.10	0.031‡
	2-Deoxy-D-glucose	0.248 ± 0.019†	0.53 ± 0.08	0.038‡**
	2-Fluoro-2-deoxy-D-glucose	0.164 ± 0.002	0.44 ± 0.07	0.188§
	D-Glucosamine	1.32 ± 0.100†	21.34 ± 3.68	2.0‡
	N-Acetyl-D-glucosamine	0.156 ± 0.017	11.11 ± 1.78	0.519§
C-3	3-Deoxy-D-glucose	13.4 ± 0.930	> 250	> 50§
	3-Fluoro-3-deoxy-D-glucose	7.24 ± 0.130	2.31 ± 0.24	> 50§
C-4	D-Galactose	61 ± 7.500†	> 250	33§
C-5	5-Thio-D-glucose	0.482 ± 0.025	11.67 ± 0.94	0.419§
C-6	D-Fructose	0.338 ± 0.016†	2.56 ± 0.40	2.54§**
	6-Deoxy-D-glucose	2.04 ± 0.270	1.54 ± 0.28**	7.3§
	6-Chloro-6-deoxy-D-glucose	1.96 ± 0.110	0.68 ± 0.09	1.7§
	6-Fluoro-6-deoxy-D-glucose	0.827 ± 0.022	–	0.314§

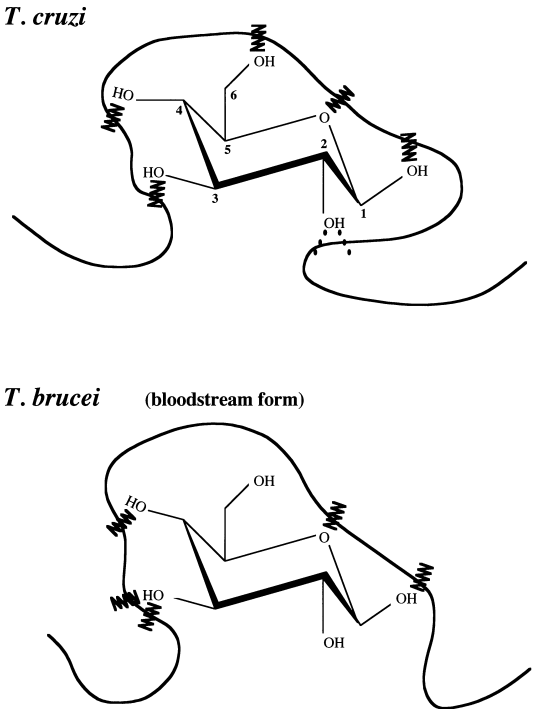

Figure 2 Proposed model of interaction between D-glucose and the hexose transporters of *T. cruzi* and *T. brucei*

This model is based on that of Eisenthal et al. [1] for *T. brucei* bloodstream forms. H-bonds between the positions C-1, C-2, C-3, C-4, C-6 and the ring oxygen with the glucose transporter are indicated. The hydrogen bonds between the less interactive hydroxyl group of the C-2 carbon with the carrier are indicated by a dotted line.

inhibitors of radiolabelled D-glucose transport. This approach reveals a difference in H-bonding patterns between the *T. brucei* and *T. cruzi* transporters (Table 1).

The C-1 analogue (1-fluoro-1-deoxy-D-glucose) inhibited D-glucose uptake very poorly ($K_i = 1.39 \pm 0.06$ mM). This suggests that there is hydrogen bonding to the oxygen of the C-1 hydroxyl of D-glucose, as seen for the procyclic but not the bloodstream form of *T. brucei* (Table 1) [1].

2-DOG was recognized by the transporter with 4-fold less affinity than D-glucose, and the addition of a fluoride residue in place of the oxygen at position C-2 restored some of the activity for the *T. cruzi* transporter. D-Glucosamine was recognized with an affinity 20-fold lower than D-glucose, although neutralization of the charge donated by an amino group, as in *N*-acetyl-D-glucosamine, restored affinity to about half that of D-glucose. In *T. brucei*, D-glucosamine also binds to both the bloodstream and procyclic transporters with an affinity 20-fold lower than D-glucose, but in these cases addition of an acetyl group only restores recognition to an affinity 10-fold lower than glucose (Table 1). The additional increase in affinity for *N*-acetyl-D-glucosamine of the *T. cruzi* transporter could result from a liaison between this sugar and the carrier protein via a hydrogen bond involving the NH group of the *N*-acetyl amine, or the carbonyl group.

Neither 3-deoxy-D-glucose, 3-fluoro-3-deoxy-D-glucose nor D-galactose significantly inhibited D-glucose uptake. This suggests that the C-3 and C-4 hydroxyls in the D-glucose configuration are essential for interaction with the transporter. Moreover, D-[1-^{14}C]galactose is not assimilated by *T. cruzi* epimastigotes (results not shown), confirming the absence of recognition of this sugar. Previously it has been reported that D-galactose was transported by *T. cruzi* [27,28]; this anomaly has probably arisen because supplies of galactose are often contaminated with D-glucose.

5-Thio-D-glucose shows an apparent K_i of 0.482 ± 0.025 mM, indicating that the ring oxygen is not absolutely essential for recognition, as seen for bloodstream and procyclic forms of *T. brucei*, although a role in H-bonding is likely.

In contrast to the bloodstream form of *T. brucei*, the C-6 hydroxyl is important for recognition by the *T. cruzi* transporter. 6-Chloro-6-deoxy-D-glucose and 6-fluoro-6-deoxy-D-glucose inhibited D-glucose uptake more than 6-deoxy-D-glucose, indicating that electronegative chlorine or fluorine atoms at this position partially, but not totally, restore H-bonding. An additional bond to the hydrogen of the C-6 hydroxyl is therefore probable.

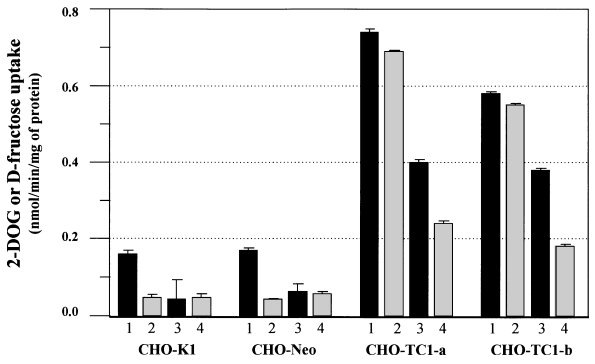

Figure 3 2-DOG and D-fructose uptake in control and transfected CHO cells

CHO-K1 control and transfected CHO cells lines were assayed for 2-DOG and D-fructose uptake as described in the Materials and methods section. CHO-K1, control non-transfected; CHO-Neo, control expressing the G-418 resistance gene; CHO-TC1-a and CHO-TC1-b, two different CHO clones transfected with the TcrHT1 gene. Values are expressed as mean ± S.D. ($n = 3$). (1) 2-DOG uptake, (2) 2-DOG uptake in the presence of 10 μM cytochalasin B, (3) D-fructose uptake, (4) D-fructose uptake in the presence of 5 mM D-glucose.

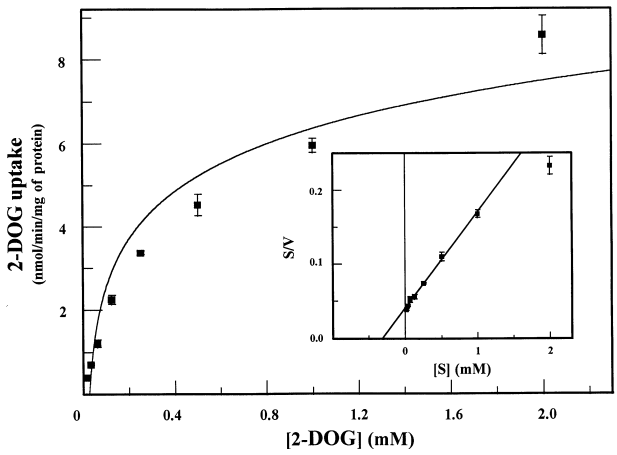

Figure 4 Kinetics of initial 2-DOG uptake in CHO cells expressing TcrHT1

The rate of 2-DOG transport was measured over the concentration range 0.031–2 mM at 22 °C in the presence of 10 μM cytochalasin B (CHO-TC1-b clone). Assays were conducted by determining the initial rate of transport (5 min time point). Values are expressed as mean ± S.D. ($n = 6$). Inset: Hanes plot.

These cumulative data fit a model of interaction between the *T. cruzi* transporter and D-glucose which may be compared with that for the bloodstream-form *T. brucei* transporter (Figure 2). Positions C-3 and C-6 are more important for the *T. cruzi* transporter in hydrogen bonding. Recognition by the *T. cruzi* transporter is more stringent than that by the bloodstream-form *T. brucei* transporter, and the H-bonds between the transporter and position C-6, in particular, may play a role in the overall higher substrate affinity.

Overexpression of the TcrHT1 transporter in CHO cells

We have previously shown that the TcrHT1 gene expressed in *Xenopus* oocytes encodes a glucose transporter [21], although kinetic measurements were not determined in this system. A second system has been developed using CHO-K1 cells to obtain a stable line expressing the TcrHT1 gene. Several mammalian glucose transporters (GLUT1, GLUT2, GLUT4) [29–31] and one trypanosomatid transporter (THT2) [2] have been successfully expressed in these cells, and the exogenously expressed proteins retain the kinetic characteristics found in the cell-types in which they are normally expressed, using suitable analogues of D-glucose. CHO-K1 is a line with relatively low levels of endogenous glucose transport activity, enhancing its utility as a system for the exogenous expression of other transporters [32].

Plasmid pCHO-TcrHT1 was transfected into CHO-K1 cells. After selection by G-418, the resultant clones were assayed for their ability to transport 2-DOG in the presence or absence of 10 μM cytochalasin B (which inhibits the endogenous but not the trypanosome transporter) and to transport D-fructose (which is a substrate for the TcrHT1 transporter but not the endogenous transporter). Two positive clones were isolated (Figure 3). Each clone showed an increase (3- to 4-fold) in hexose uptake activity compared with non-transfected CHO-K1 cells and control cells transfected with the expression vector only. Northen blot analysis using the TcrHT1 gene as a probe revealed that RNA corresponding to the gene was present in the positive lines (results not shown).

Kinetic parameters for 2-DOG uptake were determined in the presence of 10 μM cytochalasin B (Figure 4), which inhibits the endogenous transporter. An unsaturable component to the system was observed over the substrate range used. It is likely that this was due to uptake via an endogenous GLUT transporter at higher 2-DOG concentrations, when the influence of 10 μM cytochalasin B as a competitive inhibitor is correspondingly

Table 2 Percentage inhibition of 2-DOG and D-glucose uptake by D-glucose analogues

The concentration of inhibitors used for 2-DOG uptake in CHO-TC1-b was 5 mM and that for D-glucose uptake in *T. cruzi* was 0.5 mM. Values are expressed as mean ± S.D. ($n = 4$).

Inhibitors	Percentage inhibition	
	0.1 mM 2-DOG (CHO-TC1-b)	0.01 mM D-Glucose (*T. cruzi* epimastigote)
D-Glucose	86.2 ± 2.4	80.7 ± 3.4
L-Glucose	1.3 ± 7.0	7.7 ± 4.3
D-Fructose	42.6 ± 3.4	55.5 ± 7.4
D-Mannose	84.9 ± 3.0	76.9 ± 4.0
1-Fluoro-1-DOG	31.9 ± 1.9	7.4 ± 4.3
2-Fluoro-2-DOG	86.4 ± 0.5	86.5 ± 1.6
3-DOG	15.5 ± 2.3	2.0 ± 5.1
3-Fluoro-3-DOG	21.7 ± 1.7	8.2 ± 5.0
D-Galactose	2.0 ± 4.2	14.2 ± 3.7
5-Thio-D-glucose	83.3 ± 1.5	63.0 ± 2.9
6-DOG	25.2 ± 2.1	9.1 ± 10.2
6-Chloro-6-DOG	29.7 ± 0.6	7.4 ± 9.7
6-Fluoro-6-DOG	56.2 ± 1.4	62.8 ± 12.7
Cytochalasin B		
10 μM	4.4 ± 2.4	8.3 ± 5.5
150 μM	62.0 ± 1.1	62.5 ± 3.4
300 μM	72.9 ± 1.1	88.5 ± 1.6
Phloretin		
5 μM	1.3 ± 2.0	3.6 ± 5.6
150 μM	43.6 ± 2.1	43.2 ± 6.7
300 μM	77.4 ± 3.0	72.1 ± 2.7

Table 3 Effect of amino-acid-modifying agents on glucose transport

The concentration of D-glucose used for the protection was 30 mM. Values are expressed as mean ± S.D. ($n = 4$). Abbreviation: ND, not determined.

		Percentage inhibition		
Reagent (mM)	Selective for	0.5 mM D-Glucose (*T. cruzi*)	0.1 mM 2-DOG (CHO-TC1-b)	0.1 mM 2-DOG (CHO-TC1-b) + D-glucose
NEM	Cysteine			
0.1	(SH)	3 ± 9	11 ± 4	25 ± 1
0.5		23 ± 3	24 ± 5	42 ± 2
1		37 ± 2	27 ± 5	45 ± 5
PG	Arginine			
0.5		9 ± 5	20 ± 9	0 ± 1
2.5		38 ± 2	35 ± 15	0 ± 5
5		94 ± 2	ND	ND
DEP	Histidine			
0.1		31 ± 4	15 ± 9	5 ± 7
0.5		47 ± 5	59 ± 3	49 ± 2
3		69 ± 2	60 ± 2	52 ± 3
NBS	Tryptophan			
0.1		33 ± 5	2 ± 1	0 ± 2
0.5		Dead	ND	ND
EAI	Lysine			
1	(NH_2)	4 ± 6	8 ± 1	0 ± 4
5		0 ± 2	8 ± 2	0 ± 2
TNBS	Lysine			
1	(NH_2)	0 ± 2	12 ± 1	0 ± 4
5		2 ± 12	0 ± 8	2 ± 4

lower. We tested this directly by measuring the inhibition afforded by 10 μM cytochalasin B of the transporter over a range of substrate concentrations. At 4 mM 2-DOG, 10 μM cytochalasin B inhibited transport in CHO-K1 cells only 50 %, rising to 80 % inhibition when 50 μM 2-DOG was used as substrate (results not shown).

A K_m of 0.315 ± 0.01 mM with V_{max} of 7.7 ± 0.2 nmol/min per mg of protein was measured, using concentrations of 2-DOG below 1 mM to circumvent the technical difficulty imposed by the endogenous transporter. The K_m value is very similar to that measured in *T. cruzi*, indicating that the TcrHT1 gene encodes the epimastigote transporter.

We have also studied the specificity of TcrHT1 gene expressed in CHO cells by measuring the percentage inhibition of 2-DOG uptake in the presence of an excess concentration (5 mM) of various competitors and inhibitors (Table 2). The TcrHT1 transporter expressed in CHO cells showed a qualitatively similar substrate recognition profile to the transporter expressed in *T. cruzi*. Most notably, neither C-3 analogues nor D-galactose inhibited transport and 6-DOG and 1-fluoro-1-deoxy-D-glucose showed relatively poor affinity, as seen in *T. cruzi* epimastigotes. Inhibitors active against the glucose transporter in epimastigotes also inhibited the TcrHT1 transporter expressed in CHO cells. These results further support the hypothesis that the TcrHT1 gene encodes the glucose transporter measured in *T. cruzi* epimastigotes. Similar results were obtained for both clones, which differed slightly in the expression levels of TcrHT1.

Effect of amino-acid-modifying reagents on hexose transport

Having established the features of the substrate that are critical to catalytic activity, we moved on to analyse amino acid residues which may be important in substrate recognition by chemical modification of the transporter in both *T. cruzi* and CHO cells expressing the TcrHT1 gene (Table 3). Glucose transport was reduced in both systems by reagents which modify thiol groups (NEM), arginine residues (PG), and histidine residues (DEP). Apparent inhibition by NBS (which specifically modifies tryptophan residues) in parasites was not confirmed in CHO cells. This was due to a selective toxicity of NBS to the parasites under the conditions used, hence the apparent inhibition seen at 0.1 mM results from cell death rather than specific inhibition, as witnessed by assessing motility with phase-contrast microscopy. Chemical modification of amino groups using EAI and TNBS had no effect on glucose transport, ruling out a role for lysine residues in transport.

Technical limitations associated with the centrifugation-through-oil technique prohibited direct analysis of the role of substrate in protecting against this inhibition in parasites. However, it was possible to test the effects of substrate protection in the CHO cell system. Protection by D-glucose was observed in the case of arginine modification (PG), but not modification of other amino acids.

DISCUSSION

In a previous study, we characterized a glucose transporter encoded by the TcrHT1 gene from *T. cruzi* and tentatively concluded that it encodes the transporter identified in both epimastigote- and extracellular bloodstream-form trypomastigotes of this important human pathogen [21]. Kinetic parameters for 2-DOG transport and substrate specificity of the protein encoded by this gene, expressed in CHO cells, were indistinguishable from those of *T. cruzi* epimastigotes. This confirms that the TcrHT1 gene encodes the plasma membrane glucose transporter of *T. cruzi*.

The secondary structure of all members of the glucose transporter superfamily is conserved, bearing 12 putative transmembrane hydrophobic domains. Residues present in helix seven have been implicated in the recognition of substrate [33]. Inspection of the aligned sequences of the trypanosomatid transporters reveals a great sequence similarity in this region, although the *T. cruzi* transporter has a striking change in that Ser-321 replaces an otherwise totally conserved alanine residue (VXSGTLQLTGINAVMNYAPXI) [21].

Biochemically, the transporter of *T. cruzi* differs from those of other kinetoplastids in that it recognizes 2-DOG with 4-fold reduced affinity compared with D-glucose. D-Mannose, the C-2 anomer of D-glucose, is recognized with higher affinity than D-glucose itself. This would imply that an additional hydrogen bond is donated by the transporter to the C-2 oxygen of glucose. Since serine is capable of donating hydrogen bonds whereas alanine is not, it seemed possible that this biochemical difference could be attributed to Ser-321.

To date, the residues involved in binding substrate by hexose transporters in trypanosomes are unknown. The thiol-modifying reagent NEM inhibited glucose transport in both bloodstream-form and procyclic *T. brucei* [34], as well as in *Leishmania donovani* [35]. The chemical modification of cysteine residues in many proteins, however, inhibits catalytic activity without necessarily implying a role for these residues in substrate binding. The *T. cruzi* hexose transporter was also inhibited by NEM. Excess quantities of substrate, D-glucose, however, did not protect against inhibition by NEM, suggesting that cysteines located outside the substrate-binding site are modified, and that their modification affects transport independently of substrate

binding. Histidine residues seem to have a similar role in glucose transport, in that modification of these residues inhibits transport although substrate does not protect. The utility of the CHO system in analysing side-chain-modifying reagents is highlighted by the fact that the tryptophan-modifying reagent, NBS, is toxic to parasites but not to the mammalian cells under the conditions used. A role for tryptophan in the substrate-binding site has therefore also been excluded using the CHO system. Substrate does, however, protect against the inhibition afforded by the arginine-specific modifying reagent PG; hence one or more arginine residues appear to be associated with the substrate-binding site.

Comparison of the different sequences of known kinetoplastid hexose transporters (TcrHT1, THT1, THT2, Pro-1, D2) [14,16,20,21] revealed five conserved arginine residues. Of these, two were located at the extracellular face of the cell according to the classical model of glucose transporter transmembrane orientation [36,37]. The residues were located between transmembrane helices 3/4 and 5/6. One of these two residues (located between transmembrane helices 3/4) is highly conserved in other members of the glucose transporter superfamily, comprising transporters from bacteria, lower and higher eukaryotes [38] and may be generally critical in substrate binding.

Of paramount importance is the development of new drugs for use in the chemotherapy of Chagas' disease caused by *T. cruzi* and other diseases such as sleeping sickness caused by *T. brucei*. Entry of drugs into a cell is a prerequisite for intracellular toxic activity, and non-hydrophobic drugs are usually incorporated into cells by virtue of their being recognized by transport proteins, whose normal physiological substrates share recognition motifs with drugs of interest.

The lack of recognition of C-2 and C-6 hydroxyls by the *T. brucei* transporter prompted the suggestion that substituents attached to the glucose molecule at either of these two positions might be tolerated by the transporter and molecules bearing such substitutions assimilated by the cell. Addition of toxic groups would be expected to stimulate cell death.

In order to investigate atomic constituents crucial to recognition of hexoses by the *T. cruzi* transporter, we undertook a detailed analysis of the structure–activity relationship between transporter and substrate. D-Glucose and D-fructose can both act as substrates for metabolism in African trypanosomes [39,40], and the uptake of D-fructose in *T. brucei* [2,26] occurs via the same transporter as D-glucose. *T. cruzi* can also use either D-glucose or D-fructose. We have demonstrated here that the *T. cruzi* transporter is also a glucose–fructose co-transporter, in contrast to the mammalian erythrocyte transporter GLUT1 which does not accept fructose as a substrate [33]. Other mammalian hexose transporters such as GLUT2 [41] and GLUT5 [42] can transport fructose, although they recognize this product with a vastly reduced affinity when compared with the *T. cruzi* transporter. Glucose–fructose co-transport appears to be a conserved feature of kinetoplastid hexose transport molecules.

Structural analogues of D-glucose substituted at each of the six carbon positions and replacing the ring oxygen with a sulphur were tested for their ability to block 2-DOG and D-glucose transport, in order to gain further insight into components of the hexose ring critical for recognition. A similar study was performed for the *T. brucei* bloodstream-form [1] and procyclic-form transporters [2]. This study revealed significant differences between bloodstream-form *T. brucei* on the one hand and procyclic-form *T. brucei* and epimastigote-form *T. cruzi* on the other, relating to positions C-3 and C-6. The transporter of bloodstream-form *T. brucei* does not recognize the C-6 hydroxyl group oxygen, whereas this atom appears to accept hydrogen bonds from the *T. cruzi* and *T. brucei* procyclic-form transporters. Glucose molecules substituted at C-6, therefore, might be useful in the treatment of African sleeping sickness, but unfortunately not Chagas' disease. It is possible that the loss of recognition of the C-6 oxygen by the *T. brucei* bloodstream-form transporter is responsible for the significantly reduced affinity for substrate displayed by this molecule.

The capacity of these molecules to transport D-fructose with a high affinity compared with mammalian hexose transporters may represent a more useful means of developing toxic molecules specific for kinetoplastids [26].

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