

Trypanosoma evansi is alike to *Trypanosoma brucei brucei* in the subcellular localisation of glycolytic enzymes

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Trypanosoma evansi, which causes surra, is descended from *Trypanosoma brucei brucei*, which causes nagana. Although both parasites are presumed to be metabolically similar, insufficient knowledge of *T. evansi* precludes a full comparison. Herein, we provide the first report on the subcellular localisation of the glycolytic enzymes in *T. evansi*, which is alike to that of the bloodstream form (BSF) of *T. b. brucei*: (i) fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinase, phosphofructokinase, glucose-6-phosphate isomerase, phosphoglycerate kinase, triosephosphate isomerase (glycolytic enzymes) and glycerol-3-phosphate dehydrogenase (a glycolysis-auxiliary enzyme) in glycosomes, (ii) enolase, phosphoglycerate mutase, pyruvate kinase (glycolytic enzymes) and a GAPDH isoenzyme in the cytosol, (iii) malate dehydrogenase in cytosol and (iv) glucose-6-phosphate dehydrogenase in both glycosomes and the cytosol. Specific enzymatic activities also suggest that *T. evansi* is alike to the BSF of *T. b. brucei* in glycolytic flux, which is much faster than the pentose phosphate pathway flux, and in the involvement of cytosolic GAPDH in the NAD⁺/NADH balance. These similarities were expected based on the close phylogenetic relationship of both parasites.

Key words: detrended correspondence analysis - differential centrifugation - digitonin permeabilisation - haemoparasites - isopycnic ultracentrifugation - surra

The *Trypanosoma brucei* clade (order Kinetoplastida, family Trypanosomatidae, subgenus *Trypanozoon*) includes five genetically similar (Lai et al. 2008, Lun et al. 2010) parasites: *Trypanosoma brucei brucei*, which causes nagana in nonhuman mammals in sub-Saharan Africa (Finelle 1983), *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, which cause sleeping sickness in humans in sub-Saharan Africa (Simarro et al. 2010), *Trypanosoma equiperdum*, which causes dourine in equines worldwide (Zablotskij et al. 2003) and *Trypanosoma evansi*, which causes surra (*mal de cadeiras* in Brazil, *mal de caderas* in Argentina, *der-rengadera* in Venezuela, *murrina* in Central America) in nonhuman mammals throughout the tropics and subtropics (Desquesnes et al. 2013).

T. evansi is thought to be descended from *T. b. brucei*, from which it differs in the complete loss of kinetoplast maxicircles, which are involved in the morphological transformation and multiplication within tsetse flies, thus causing a dependence on these endemic Sub-Saha-

ran vectors (Lun & Desser 1995, Lai et al. 2008). A shift to a purely mechanical transmission through the bite of cosmopolitan genera of haematophagous flies may have allowed *T. evansi* to spread throughout the tropics and subtropics (Lun & Desser 1995). In addition, *T. evansi* differs from *T. b. brucei* in possessing: (i) a kinetoplast with one extremely predominant minicircle sequence class, as opposed to a kinetoplast with hundreds of classes (Li et al. 2006) and (ii) an ATP synthase partly lacking a F₀ domain, and having a F₁ domain hydrolysing ATP to ADP in the mitochondrial matrix and thus, together with an ATP⁴/ADP³ electrogenic transporter, participating in the maintenance of the inner mitochondrial membrane potential (Schnauffer et al. 2005).

With regard to glycolytic metabolism, both parasites are known to be alike in (i) experiencing exponential population growth in the bloodstream of rats and mice in the course of infection (Queiroz et al. 2000, Seed & Wenck 2003), (ii) reducing the blood glucose concentration in infected mammals during such growth (Seed & Wenck 2003, Abdelrahman et al. 2004, Albert et al. 2005, Cadioli et al. 2006, Kumar et al. 2009), (iii) generating pyruvate through glucose consumption as the main product under aerobic conditions (Marshall 1948, Ryley 1956, Grant & Fulton 1957, Haanstra et al. 2012) and (iv) lacking dependence on oxidative phosphorylation to produce ATP during glucose consumption (Evans & Brown 1971, Njogu et al. 1980, Lai et al. 2008).

A substantial gap in the knowledge of *T. evansi* precludes a full comparison with the metabolism of *T. b. brucei*. For example, the energetic balance of glycolysis and the structural and kinetic properties of glycolytic enzymes and their inhibitors have not been studied in *T.*

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evansi. These enzymes are of special interest as potential targets for therapeutic drugs to treat African trypanosomiasis (Verlinde et al. 2001, Haanstra et al. 2011). Our objective is to reduce this gap by determining for the first time whether *T. evansi* is alike to the bloodstream form (BSF) of its close relative, *T. b. brucei*, regarding the subcellular localisation and specific activities of glycolytic enzymes.

MATERIALS AND METHODS

This study was performed in accordance with the principles of laboratory animal care of our institution, the Venezuelan laws and the international laws, such as the EU Directive 2010/63/EU for animal experiments.

Our stock of *T. evansi* (TeAp-N/D1) (Perrone et al. 2009) originated from horses in the Venezuelan Llanos region, where equine trypanosomiasis is endemic (Moreno et al. 2013). We cultured parasites in Sprague-Dawley rats inoculated intraperitoneally with 100 µL of rat blood containing live *T. b. evansi* at a concentration of 3.6×10^8 parasites/mL. We used phosphate buffer saline (PBS), pH 8.1 (Lanham & Godfrey 1970), to dilute (50%) infected rat blood samples, which we stored in liquid nitrogen until use in subsequent inoculations.

We use the following enzyme abbreviations: fructose-bisphosphate aldolase (ALD), enolase (ENO), glycerol-3-phosphate dehydrogenase (G3PDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamate-oxaloacetate transaminase (GOT), glucose-6-phosphate dehydrogenase (GPD), hexokinase (HK), lactate dehydrogenase (LD), malate dehydrogenase (MD), phosphofructokinase (PFK), glucose-6-phosphate isomerase (PGI), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), pyruvate kinase (PK) and triosephosphate isomerase (TIM).

Localisation of glycolytic enzymes in subcellular fractions - We used 20 infected rats. By means of a Neubauer haemocytometer, we estimated the concentration of parasites in rat-tail blood samples. When the density of *T. evansi* in blood reached 10^9 parasites/mL, we anaesthetised the rats using diethyl ether and sacrificed them by exsanguination using cardiac puncture with a 12 cc syringe previously containing 1 mL of PBSG (57 mM Na₂HPO₄, 3 mM NaH₂PO₄, 43.8 mM NaCl, 1% glucose at pH 8.1), to which we added 3.5% sodium citrate. We centrifuged the blood so obtained at 750 g for 10 min at ~23°C to separate the cells from the plasma. Thereafter, we washed the cell pellet twice with PBSG and eliminated most of the erythrocyte layer by suction with a Pasteur pipette connected to a low vacuum system. We resuspended the remainder of the pellet in PBSG. We passed this eluate through a diethylaminoethyl (DEAE) cellulose column previously equilibrated with PBSG (Lanham & Godfrey 1970). Because the blood cells are retained by DEAE cellulose, this process results in purified parasites contained in a new eluate, which we centrifuged at 3,000 g and 10°C for 15 min to obtain a *T. evansi* pellet which we kept in ice until use in further procedures. These involved several techniques, namely selective membrane permeabilisation with digitonin, differential centrifugation and isopycnic ultracentrifugation to obtain purified glycosomes, whose

products we evaluated through spectrophotometry and by means of western blot (WB) analysis to determine the subcellular localisation of: (i) glycolytic enzymes, (ii) the glycolysis-auxiliary enzyme G3PDH, (iii) the cytosol marker enzymes MD and GOT and (iv) the dual marker (cytosol and glycosome) enzyme GPD.

Selective membrane permeabilisation with digitonin (Steiger et al. 1980) involved re-suspension of the *T. evansi* pellet in buffer A [25 mM Tris-HCl, pH 7.4, with added sucrose and ethylenediamine tetraacetic acid (EDTA) to obtain final concentrations of 250 mM and 1 mM, respectively] to obtain a parasite protein concentration of 1 mg/mL. Thereafter, we divided the *T. evansi* suspension in 1.5 mL Eppendorf tubes and added varying amounts (ranging from 0.01–0.6 mg) of digitonin. After incubating the tubes for 30 min, we centrifuged them at 14,000 g for 2 min. After washing them with buffer A, we resuspended the resulting pellets in 200 µL buffer A containing 0.1% v/v of Triton X-100. We determined spectrophotometrically the activity of the enzymes of interest in the supernatant. In addition, we used the pellets and their corresponding supernatants to test for the presence of HK using WB analysis.

Differential centrifugation (Steiger et al. 1980, Taylor & Gutteridge 1987) involved: (i) washing the *T. evansi* pellet twice with buffer A, (ii) mixing the pellet at a 1:1 proportion with 200 mesh silicate carbide, (iii) adding buffer B (10 mM HEPES pH 7.2, 250 mM sucrose, 25 mM NaCl, 2 mM Na₂EDTA and 5 mM dithiothreitol) containing a protease inhibiting cocktail (57 µM PMSF, 100 µM TLCK, 10 µM leupeptin and 1 mM benzamide), (iv) breaking the mixture through abrasion in a mortar at 4°C, (v) centrifuging the mixture at 150 g for 3 min at 4°C to eliminate the resulting silicate carbide sediment, (vi) centrifuging the supernatant at a 1,000 g for 10 min at 4°C to obtain an unbroken, parasite and nuclei-rich pellet [nuclear fraction (NF)] and a supernatant, (vii) centrifuging the supernatant at a 5,000 g for 15 min at 4°C to obtain a mitochondrial and large cellular debris [large granular fraction (LGF)] and a supernatant, (viii) centrifuging the supernatant at 33,000 g for 20 min at 4°C to obtain a glycosome-rich pellet [small granular fraction (SGF)] and a supernatant, (ix) ultracentrifuging the supernatant at 105,000 g for 90 min at 4°C to obtain a microsomal pellet [microsomal fraction (MF)] and a supernatant corresponding to the cytosol [cytosolic fraction (CF)], (x) washing the pellets (NF, LGF, SGF, MF) with buffer A and solubilising them in 150 mM of NaCl containing 0.1% Triton X-100, (xi) for NF, LGF, SGF, MF and CF, using Lowry assays to determine protein concentrations (to eliminate potential contaminants, we precipitated the proteins before these assays using 10% trichloroacetic acid) (Rosenberg 2005), (xii) for CF and solubilised NF, LGF, SGF, MF, spectrophotometrically determining the specific activity (*sa*) of the enzymes of interest and (xiii) for CF and solubilised NF, LGF, SGF, testing for the presence of HK using WB analysis.

To visualise the degree of association of enzymes, as measured by their specific activities, with each other and with the subcellular fractions resulting from differential centrifugation, we used detrended correspondence

analysis (DCA). This multivariate statistical technique is an improved variant (Hill & Gauch Jr 1980) of correspondence analysis, a visualisation method picturing the association of rows (in our case enzymes) and columns (in our case subcellular fractions) of a contingency table in a single low-dimensional space, representing the most important relationships (Sourial et al. 2010). For the needed calculations, we used Paleontological Statistics Software Package for Education and Data Analysis v.3.04 (Hammer et al. 2001).

Presence of glycolytic enzymes in purified glycosomes

- Purification of glycosomes by means of isopycnic ultracentrifugation (Oppendoes et al. 1984) involved layering the SGF (step 8 of the previously described experiment) onto a linear 0.3-2.5 M sucrose gradient containing buffer A, followed by centrifugation at 170,000 g for 2 h at 4°C. Thereafter, we removed the layer corresponding to highly purified glycosomes (equilibrated at 1.23 g/cm³) by puncturing the side of the tube with a syringe, divided this layer into two fractions, placed one of them in a 150 mM of NaCl solution containing 0.1% Triton X-100 and determined spectrophotometrically the activity of the enzymes of interest in both fractions. In addition, we tested for the presence of HK by means of WB analysis. We estimated the latency of HK and PGK using the relation $l = [(a - b)/a] \times 100$, where l = percent latency, a = enzyme activity of glycosomes in 0.1% Triton X-100 and b = enzyme activity outside glycosomes (Steiger et al. 1980).

We spectrophotometrically assayed the enzymes in quartz cuvettes at ~23°C by detecting the oxidation/reduction of NADPH/NADP⁺ and NADH/NAD⁺ at 340 nm in a final volume of 1 mL. One unit (U) of enzyme activity is defined as the amount of the enzyme required to catalyse the formation (transformation) of 1 µmol/min of product (substrate). We followed the procedures of Bergmeyer (1983) for GOT, GPD, MD and PK, Misset & Oppendoes (1984) for ALD, G3PDH, GAPDH, HK, PFK, PGI, PGK and TIM [in the case of PFK we measured the enzymatic activity in absence of allosteric activator (AMP)], Durany et al. (1996) for PGM (the reaction mix contained 100 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM MgCl₂, 1 mM ADP, 4 mM 3-phosphoglycerate, 0.2 mM NADH, 1.4 U/mL ENO, 1.4 U/mL PK and 5 U/mL LD) and Hannaert et al. (2003b) for ENO.

RESULTS

Localisation of glycolytic enzymes in subcellular fractions - Three enzyme groups were distinguished according to their release patterns after selective membrane permeabilisation with digitonin (Fig. 1): (i) MD and ENO, reaching 100% activity at 0.04-0.08 mg/mg of digitonin/protein, thus these enzymes are cytosolic (ENO is a marker enzyme for the cytosol) (Hannaert et al. 2003b), (ii) G3PDH, PGK and HK, reaching 100% activity at 0.18-0.25 mg/mg of digitonin/protein, thus these enzymes are glycosomal (HK is a marker enzyme for glycosomes) (Oppendoes & Borst 1977, Hart et al. 1984, Misset & Oppendoes 1984, Oppendoes et al. 1984, Hammond et al. 1985, Misset et al. 1986) and (iii) PGI and GPD, with a biphasic pattern, thus these enzymes

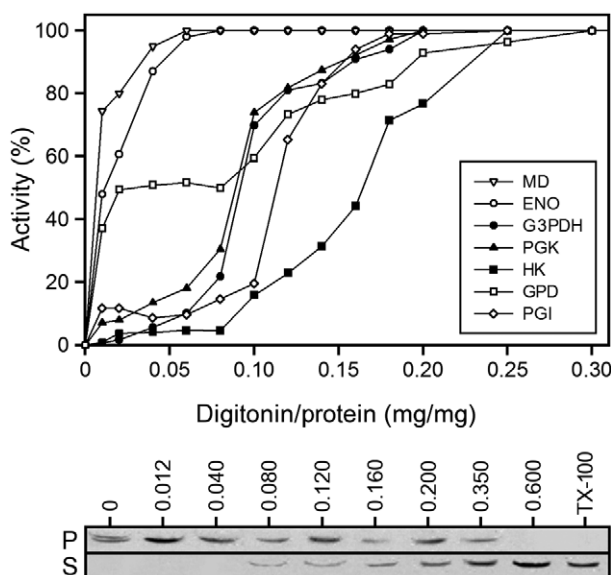


Fig. 1: subcellular distribution of percent activities of seven enzymes in *Trypanosoma evansi* determined by selective membrane permeabilisation with digitonin. Top (activity graph): release of enzymes after treatment with several concentrations of digitonin; bottom (scan of polyvinylidene difluoride membrane): western blot analysis (40 µg of protein in each well) of pellet (P) and supernatant (S), using antisera against trypanosome hexokinase (HK), for several digitonin concentrations. The last well (TX-100) was loaded with Triton X-100 (0.1%) solubilised parasite cells. ENO: enolase; G3PDH: glycerol-3-phosphate dehydrogenase; GPD: glucose-6-phosphate dehydrogenase; MD: malate dehydrogenase; PGI: glucose-6-phosphate isomerase; PGK: phosphoglycerate kinase.

are both cytosolic and glycosomal. In the case of PGI, 12% of its activity was reached at 0.01-0.02 mg/mg of digitonin/protein and 100% of its activity was reached at 0.18-0.25 mg/mg of digitonin/protein. In the case of GPD, 50% of its activity was reached at 0.02-0.04 mg/mg of digitonin/protein and 100% of its activity was reached at 0.25-0.30 mg/mg of digitonin/protein. To confirm the HK release pattern, we applied WB and immunodetection to pellets and supernatants obtained during digitonin permeabilisation of the parasites (Fig. 1). The HK signal disappeared from the pellet at digitonin/protein levels greater than 0.35 mg/mg and became decidedly stronger in the supernatant at digitonin/protein levels \geq 0.20 mg/mg. Recall that HK was in the second enzyme group distinguished above for which 100% activity was reached at 0.18-0.25 mg/mg of digitonin/protein.

Three enzyme groups were distinguished according to their distributional patterns in the subcellular fractions obtained through differential centrifugation (Fig. 2): (i) HK, PGK, PFK, G3PDH, ALD, PGI and TIM, with maximum *sa* in the glycosome-rich pellet (SGF), (ii) GAPDH and GPD, with high specific activities in both the glycosome-rich pellet and the supernatant (CF), and (iii) ENO, MD, GOT, PK and PGM, with maximum *sa* in the supernatant. To confirm the distributional pattern of HK through differential centrifugation, we applied WB analysis and immunodetection (Fig. 2, top

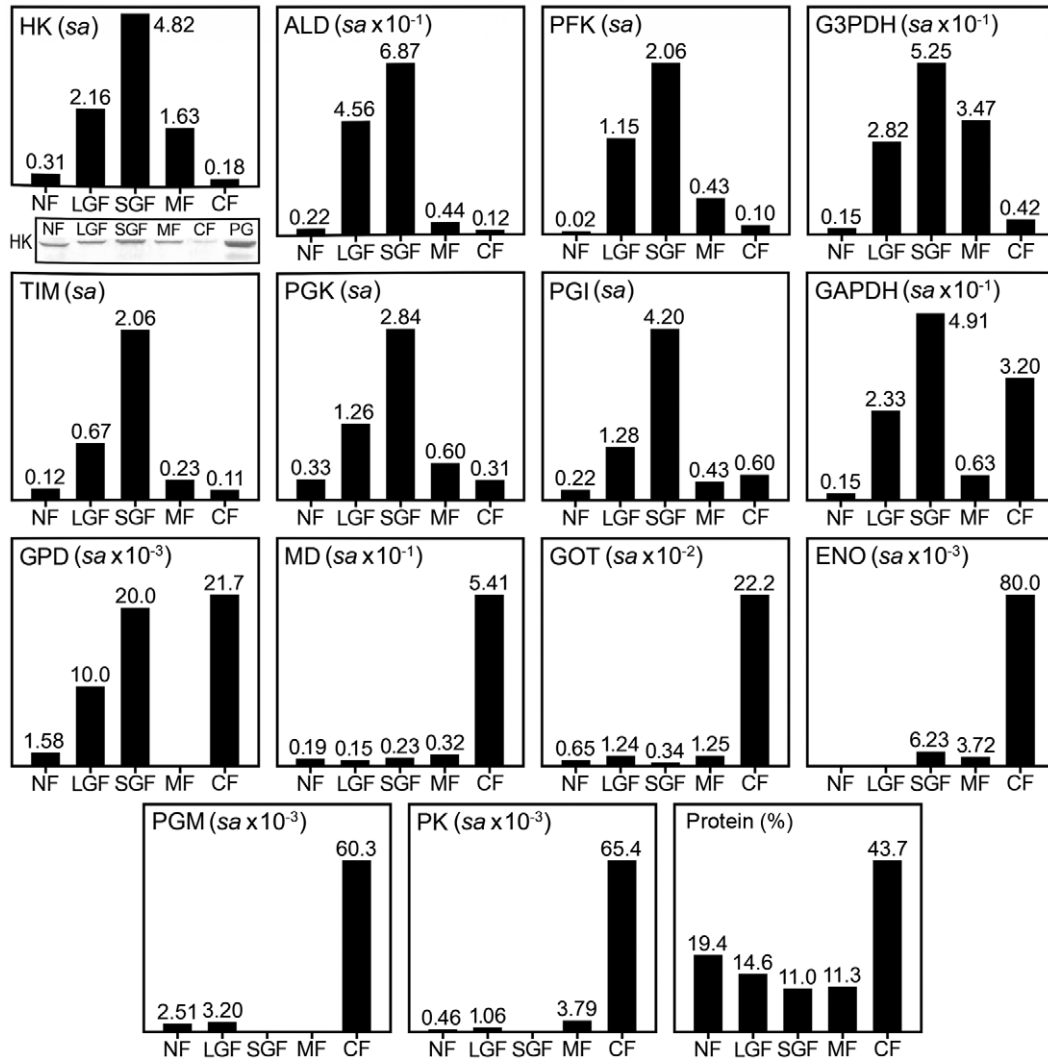


Fig. 2: distribution of specific activities of 14 enzymes and percent protein (total = 298.5 mg) in five *Trypanosoma evansi* subcellular fractions resulting from differential centrifugation. The polyvinylidene difluoride membrane scan (top left) corresponds to the western blot (40 µg of protein in each well) of fractions and purified glycosomes (PG) using antisera against trypanosome hexokinase (HK). ALD fructose-bisphosphate aldolase; CF: cytosolic fraction; ENO: enolase; G3PDH: glycerol-3-phosphate dehydrogenase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GOT: glutamate-oxaloacetate transaminase; GPD: glucose-6-phosphate dehydrogenase; LGF: large granular fraction; MD: malate dehydrogenase; MF: microsomal fraction; NF: nuclear fraction; PFK: phosphofructokinase; PGI: glucose-6-phosphate isomerase; PGK: phosphoglycerate kinase; PGM: phosphoglycerate mutase; PK: pyruvate kinase; sa: specific activity; SGF: small granular fraction; TIM: triosephosphate isomerase.

left). As expected, the HK signal was highest in SGF and weakest in CF. The rupture of some glycosomes might account for the weak HK signal in CF.

Multivariate analysis (Fig. 3) confirmed the visual observation (Fig. 2) of a group of primarily glycosomal enzymes (ALD, G3PDH, HK, PFK, PGI, PGK, TIM), a group of primarily cytosolic enzymes (ENO, GOT, MD, PGM, PK) and a group of simultaneously glycosomal and cytosolic enzymes (GAPDH, GPD). Multivariate analysis (Fig. 3) also indicated that glycosomal enzymes are most closely associated with LGF and SGF, that cytosolic enzymes are almost exclusively associated with CF and that most enzymes are not or are weakly associated with NF and, particularly, with MF. The eigenvalue

(0.3315) for axis 1 was much higher than the eigenvalue (0.0237) for axis 2 (eigenvalues for axes 3 and 4 were 0.0088 and 0.0016, respectively), indicating that the transition from LGF and SGF to CF, represented by axis 1, is more important than the transition from LGF, SGF and CF to MF, represented by axis 2 (Fig. 3). Therefore, axis 1 was sufficient to represent enzyme concomitances along the examined fractions.

Presence of glycolytic enzymes in purified glycosomes - The results obtained using selective membrane permeabilisation with digitonin and differential centrifugation suggest the presence of eight enzymes in glycosomes. To corroborate this result, we evaluated the sa of these enzymes in purified glycosomes obtained through

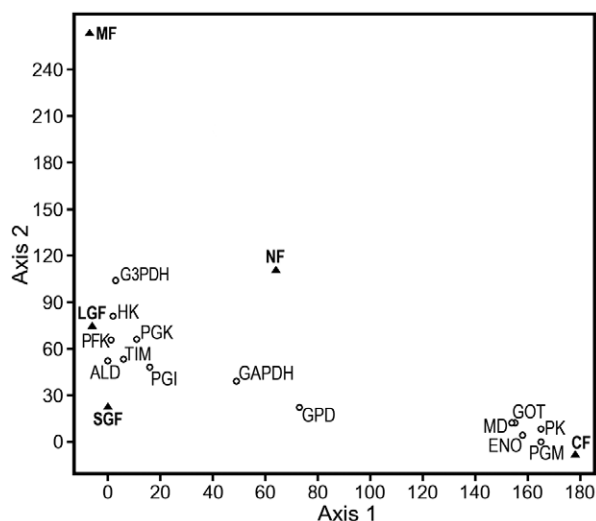


Fig. 3: detrended correspondence analysis of differential centrifugation data (Fig. 2): relative position of 14 enzymes (open circles) and five subcellular fractions (solid triangles) in the plane defined by axes 1 and 2. ALD: fructose-bisphosphate aldolase; CF: cytosolic fraction; ENO: enolase; G3PDH: glycerol-3-phosphate dehydrogenase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GOT: glutamate-oxaloacetate transaminase; GPD: glucose-6-phosphate dehydrogenase; HK: hexokinase; LGF: large granular fraction; MD: malate dehydrogenase; MF: microsomal fraction; NF: nuclear fraction; PFK: phosphofructokinase; PGI: glucose-6-phosphate isomerase; PGK: phosphoglycerate kinase; PGM: phosphoglycerate mutase; PK: pyruvate kinase; SGF: small granular fraction; TIM: triosephosphate isomerase.

isopycnic ultracentrifugation. The mean *sa* values (U/mg) measured in purified glycosomes are shown in Table. In addition, we used WB analysis to corroborate the presence of HK in purified glycosomes (Fig. 2, top left). The latency of HK is 90% and that of PGK is 85%.

DISCUSSION

Localisation of glycolytic enzymes in subcellular fractions - *T. evansi* (this study) and *T. b. brucei* (previous studies) are similar in several enzyme release patterns determined through selective membrane permeabilisation with digitonin (Fig. 1). In *T. evansi*, ENO and MD are fully released at a digitonin concentration (0.04 mg/mg digitonin/protein) similar to that required for the permeabilisation of the *T. b. brucei* cell membrane (Visser & Opperdoes 1980, Besteiro et al. 2002), suggesting that these enzymes are cytosolic in both parasites. In *T. evansi*, G3PDH, PGK and HK are fully released at a digitonin concentration (0.20 mg/mg) similar to that required for the permeabilisation of the *T. b. brucei* glycosomes (Besteiro et al. 2002), suggesting that these enzymes are glycosomal in both parasites. We observed that the release of G3PDH and PGK started at a slightly lower digitonin concentration than HK, suggesting that the latter is part of a crystalloid nucleus, as proposed for *T. b. brucei* (Misset et al. 1986). If G3PDH and PGK were not nuclear, then higher solubilities compared with HK would be expected. In *T. evansi*, PGI and GPD were released at two different digitonin concentrations, i.e., the concentrations required to permeabilise the cell

TABLE

Comparison of *Trypanosoma evansi* (this study) and the bloodstream form of *Trypanosoma brucei brucei* (previous studies) regarding specific activity (*sa*) values^a of eight enzymes found in homogenate and glycosomes

Enzyme	n	H	G	G/H
<i>Trypanosoma evansi</i>				
ALD	1	0.044	0.6	13.6
G3PDH	1	0.19	1	5.3
GAPDH	1	0.16	0.6	3.8
HK	3	0.93 (0.70-1.20)	6.2 (5.18-7.91)	6.7
PFK	1	0.38	2.3	6.1
PGI	2	0.64 (0.52-0.76)	4.7 (4.2-5.2)	7.3
PGK	3	1.03 (0.59-1.53)	4.7 (3.85-6.22)	4.6
TIM	1	0.72	3.22	4.5
<i>Trypanosoma brucei brucei</i> ^b				
ALD	4	0.27 (0.02-0.81)	3.13 (0.8-8.9)	11.6
G3PDH	5	0.61 (0.3-1.3)	6.18 (2.5-8.2)	10.1
GAPDH	5	0.19 (0.098-0.42)	3.54 (1.2-10.2)	18.6
HK	5	0.81 (0.63-1.3)	8.03 (2.5-9.91)	10
PFK	5	1.10 (0.75-2.0)	8.53 (2.8-18)	7.8
PGI	5	0.94 (0.78-1.06)	7.82 (3-10.32)	8.3
PGK	5	0.66 (0.24-0.88)	4.74 (2.7-5.8)	7.2
TIM	5	4.07 (0.92- 10)	12.73 (7.71-16.4)	3.1

a: expressed in U/mg; b: values taken from Hart et al. (1984), Misset and Opperdoes (1984), Opperdoes et al. (1984), Hammond et al. (1985) and Misset et al. (1986); G: *sa* in glycosomes; G/H: purification factor; H: *sa* in homogenate; n: number of experiments used to obtain *sa* values [average *sa* (minimum *sa* - maximum *sa*)].

membrane and glycosomes, suggesting that, similarly to GPD in the BSF and the procyclic (PC) stage of *T. b. brucei* (Heise & Opperdoes 1999, Duffieux et al. 2000), each of these enzymes consists of one cytosolic and one glycosomal isoenzyme.

T. evansi (this study) and *T. b. brucei* (previous studies) are similar in several enzyme subcellular distribution patterns determined through differential centrifugation (Figs 2, 3). In *T. evansi*, HK, PGK, PFK, ALD, PGI, G3PDH and TIM were observed in the glycosome-rich pellet, reproducing findings for the BSF of *T. b. brucei* (Opperdoes & Borst 1977, Steiger et al. 1980). In *T. evansi*, GAPDH and GPD were both observed in the glycosome-rich pellet and in the cytosol, reproducing findings for GAPDH in the BSF (Opperdoes & Borst 1977, Steiger et al. 1980) and for GPD in the BSF and PC (Heise & Opperdoes 1999, Duffieux et al. 2000) of *T. b. brucei*. In *T. evansi*, ENO, MD, GOT, PK and PGM were observed in the cytosol, reproducing findings for the BSF of *T. b. brucei* (Opperdoes & Borst 1977, Oduro et al. 1980, Hannaert et al. 2003b, Aranda et al. 2006, Marciano et al. 2008).

Although selective membrane permeabilisation with digitonin indicated a small proportion of PGI activity to be cytosolic (PGI was the only enzyme whose subcellular localisation diverged, albeit slightly, using different methods), multivariate analysis of the differential centrifugation data (Fig. 3) clearly place this enzyme in the glycosomal group.

Presence of glycolytic enzymes in purified glycosomes - T. evansi (this study) and the BSF of *T. b. brucei* (previous authors) are similar regarding purification factors (3.8-13.8 times vs. 3.1-18.6 times with respect to the homogenate) of eight enzymes and in the specific activities of three enzymes (HK, PGI and PGK) present in the purified glycosomes obtained through isopycnic ultracentrifugation (Table). However, for some enzymes (ALD, G3PDH, GAPDH, PFK and TIM), dissimilar experimental conditions result in different *sa* values for *T. evansi* (Table). In the case of ALD, G3PDH, GAPDH and TIM, the *sa* values were inferior for *T. evansi* owing to a low ionic strength and the absence of reducing agents in the buffer (the activities of ALD and GAPDH increased five-fold under the opposite conditions) (Misset et al. 1986, 1987). In the case of PFK, the *sa* for *T. evansi* was similar to the lowest value for the BSF of *T. b. brucei* owing to the absence of AMP in the buffer (maximum activity requires the presence of AMP) (Misset & Opperdoes 1984). Notwithstanding some underestimations, the increased specific activities in purified glycosomes with respect to the homogenate of ALD, GAPDH, HK, PFK, PGI and PGK imply that these enzymes are glycosomal. As a control, we verified that there was no activity of the cytosolic enzymes ENO or PK in purified glycosomes.

Concluding remarks - Based on protein homologies, G3PDH, GAPDH, MD and TIM in *T. evansi* have been inferred to have the same localisation as in *T. b. brucei* (Roy et al. 2010). The results of two techniques (digitonin permeabilisation, differential centrifugation) implied that, as in the BSF of *T. b. brucei*, in *T. evansi*: (i) HK (marker enzyme), ALD, G3PDH, PFK, PGI, PGK and TIM are located in glycosomes, (ii) ENO (marker enzyme), GOT, MD, PGM and PK are located in the cytosol and (iii) GAPDH and GPD are located in both glycosomes and in the cytosol. A third technique (isopycnic ultracentrifugation) confirmed that, as in the BSF of *T. b. brucei*, in *T. evansi*, HK, ALD, G3PDH, GAPDH, PFK, PGI and PGK are located in glycosomes and ENO and PK are not.

For *T. evansi*, as in the BSF of *T. b. brucei* (Opperdoes 1987), we expected ATP consumption to be catalysed by HK and PFK in glycosomes, and ATP synthesis to be catalysed by PGK in glycosomes and by PK in the cytosol. Moreover, the resemblance regarding the specific activities of HK, PGI and PGK between *T. evansi* and the BSF of *T. b. brucei* (Table) suggests glycolytic flux to be alike in both. The localisation of MD (presence in cytosol, absence in mitochondria) in *T. evansi* was similar to that reported for the BSF of *T. b. brucei* suggesting lack of an operative Krebs cycle (Aranda et al. 2006).

In *T. evansi*, the function of the dual localisation of GPD, an enzyme of the oxidative phase of the pentose

phosphate pathway, might be similar to that proposed for the BSF of *T. b. brucei* (Duffieux et al. 2000), namely, synthesising the required reducing power (NADPH) in cytosol and glycosomes. In *T. evansi*, the specific activities of cytosolic and glycosomal GPD are much lower (217-310 times) than those of glycolytic enzymes (HK, PGI, PGK), suggesting a much slower flux of the pentose phosphate pathway relatively to that of glycolysis, as reported for the BSF of *T. b. brucei* (Hannaert et al. 2003a). Although it is not known whether host glucose is oxidised through the pentose phosphate pathway in *Trypanozoon*, the viability of the BSF of *T. b. brucei* depends on GPD (Cordeiro et al. 2009). Species of *Trypanozoon* transform glucose into pyruvate (62.8-96.1%), glycerol (0-29%), CO₂ (1.7-6.1%), succinate (0-2.9%), lactate (0-2.6%), acetate (0-1.7%) and citrate (0-1.2%) under aerobic conditions (Ryley 1956). Thus, the CO₂ released by these species might partly result from glucose oxidation in the pentose phosphate pathway.

Evolutionary, cytosolic and glycosomal GAPDH may have been independently acquired (Michels et al. 1991). The function of cytosolic GAPDH is unknown. In *T. evansi*, as in the BSF of *T. b. brucei* (Opperdoes & Borst 1977), glycosomal GAPDH is likely to be a glycolytic enzyme having a much lower affinity for NADH and NAD⁺ than cytosolic GAPDH (Lambeir et al. 1991). Therefore, cytosolic GAPDH should be involved in the NAD⁺/NADH balance of *T. evansi* and the BSF of *T. b. brucei*, as shown for *Leishmania donovani* (Zhang et al. 2013). The exchange of phosphorylated intermediates of glycolysis between glycosomes and the cytosol at a rate 60 times lower than glycolytic flux (Visser et al. 1981) is consistent with this hypothesis. Pore-forming channels might be involved (Gualdrón-López et al. 2012). A similar mechanism might explain how glucose-6-phosphate, catalytically produced by HK in glycosomes, can become a substrate for GPD in the cytosol in both *T. evansi* and *T. b. brucei*.

Owing to their close phylogenetic relationship and because maxicircles are not needed to thrive in blood, *T. evansi* and the BSF of *T. b. brucei* are expected to be metabolically similar (Lai et al. 2008), as our results have shown for the glycolytic pathway.

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