Anais da Academia Brasileira de Ciências (2007) 79(4): 649-663
(Annals of the Brazilian Academy of Sciences)
ISSN 0001-3765
www.scielo.br/aabc

The pentose phosphate pathway in *Trypanosoma cruzi*: a potential target for the chemotherapy of Chagas disease

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Manuscript received on August 13, 2007; accepted for publication on August 17, 2007;
presented by LUCIA MENDONÇA PREVIATO

ABSTRACT

Trypanosoma cruzi is highly sensitive to oxidative stress caused by reactive oxygen species. Trypanothione, the parasite's major protection against oxidative stress, is kept reduced by trypanothione reductase, using NADPH; the major source of the reduced coenzyme seems to be the pentose phosphate pathway. Its seven enzymes are present in the four major stages in the parasite's biological cycle; we have cloned and expressed them in *Escherichia coli* as active proteins. Glucose 6-phosphate dehydrogenase, which controls glucose flux through the pathway by its response to the NADP/NADPH ratio, is encoded by a number of genes per haploid genome, and is induced up to 46-fold by hydrogen peroxide in metacyclic trypomastigotes. The genes encoding 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase, transaldolase and transketolase are present in the CL Brener clone as a single copy per haploid genome. 6-phosphogluconate dehydrogenase is very unstable, but was stabilized introducing two salt bridges by site-directed mutagenesis. Ribose-5-phosphate isomerase belongs to Type B; genes encoding Type A enzymes, present in mammals, are absent. Ribulose-5-phosphate epimerase is encoded by two genes. The enzymes of the pathway have a major cytosolic component, although several of them have a secondary glycosomal localization, and also minor localizations in other organelles.

Key words: *Trypanosoma cruzi*, Chagas disease, pentose phosphate pathway, oxidative stress, NADPH generation.

INTRODUCTION

Trypanosoma cruzi, a flagellated protozoan parasite, is the causative agent of the American trypanosomiasis, Chagas disease. The parasite has a complex life cycle, involving two forms present in the gut of the insect vector, the replicative epimastigote and the infective metacyclic trypomastigote, and two forms present in the infected mammal, the intracellular amastigote and the bloodstream trypomastigote, released from infected cells into the blood. The infection is endemic in Latin America, where its prevalence is estimated at 16-18 million cases, with about 120 million people at risk. No vaccines have been developed so far, and the low effectiveness of the chemotherapeutic agents available (at present, only benznidazole), together with their undesirable side effects, makes treatment of Chagas disease difficult. There is, therefore, an urgent need for the identification of novel drug targets to improve the treatment of this disease (Barrett et al. 2003).

Throughout its life cycle this parasite is exposed to oxidative stress imposed by reactive oxygen species (ROS) derived from its own aerobic metabolism and from the host immune response (Docampo 1990). To detoxify hydroperoxides, *T. cruzi* possesses several pathways

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which have different substrate specificities and subcellular localization. Wilkinson et al. demonstrated the presence of two glutathione peroxidases, TcGPXI (Wilkinson et al. 2002a) and TcGPXII (Wilkinson et al. 2002b), localized in different cell compartments; the former in the cytosol and glycosomes, the latter in the endoplasmic reticulum. They also detected two trypanothione dependent members of the peroxiredoxine family TcMPX and TcCPX, (Wilkinson et al. 2000), and an ascorbate dependent hemoperoxidase TcAPX (Wilkinson et al. 2002c). These enzymes belong to an intricate network converging to reduced trypanothione $T[SH]_2$, which is maintained in its reduced form by trypanothione reductase, with the utilization of NADPH. Therefore, it is absolutely essential for the parasite to have reliable pathways for the maintenance of a suitable pool of this reduced coenzyme. Several enzyme reactions may be responsible for this supply of reducing power: the NADP-linked glutamate dehydrogenase (Juan et al. 1978, Barderi et al. 1998), the NADP-linked malic enzyme (Cannata et al. 1979), and the two dehydrogenases of the Pentose Phosphate Pathway (PPP).

In most organisms glucose is metabolized through two major pathways: the glycolytic, or Embden-Meyerhof, pathway, and the PPP. The latter one (Fig. 1), also starting from glucose 6-phosphate (G6P), is involved in the production of the ribose 5-phosphate (R5P) required for nucleotide synthesis, and of reducing power in the form of NADPH. The pathway can be separated in two branches, namely an oxidative branch, involving glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconolactonase (6-PGL) and 6-phosphogluconate dehydrogenase (6PGDH), and a non-oxidative, or sugar interconversion, branch, involving ribose 5-phosphate isomerase (RPI), ribulose 5-phosphate epimerase (RPE), transaldolase (TAL) and transketolase (TKT) (Fig. 1). The PPP has also been known as the pentose phosphate cycle, since, when functioning as a whole, the fructose 6-phosphate (F6P) and glyceraldehyde 3-phosphate (Gly3P) formed can be converted back into glucose 6-phosphate, entering the oxidative branch again. However, the PPP needs not to act as a cycle, and the different enzymatic reactions will be operative according to the cell needs (Stryer 1999).

Differing from the glycolytic pathway, which has been thoroughly studied in Trypanosomatids over the last three decades, the PPP has received much less attention up to the last decade. In the case of *T. cruzi*, early studies showed that both dehydrogenases were present in epimastigotes (Raw 1959), and one of them (G6PDH) was partially purified and some of its properties were determined (Funayama et al. 1977). Moreover, studies with labeled glucose suggested that the PPP was functional in some strains of the parasite (Mancilla and Náquira 1964). However, until recently most of the enzymes of the pathway had not been detected, and nothing was known about their properties and subcellular localization. Over the last few years, we have studied in detail this subject. This review will describe the present situation in *T. cruzi*, comparing, whenever possible, with the findings made in *Trypanosoma brucei* and *Leishmania* spp.

FUNCTIONALITY OF THE PENTOSE PHOSPHATE PATHWAY IN *Trypanosoma cruzi*

The early studies of Mancilla and Náquira (1964) suggested that the PPP was functional in two strains of *T. cruzi*. Recently we performed similar experiments with intact epimastigotes of the CL Brener clone, using glucose labeled with ^{14}C in C_1 or C_6. An increased production of $^{14}CO_2$ from $^{14}C_1$-glucose over that from $^{14}C_6$-glucose indicates that the PPP is functional (Katz and Wood 1963). From our experiments we could conclude that in normal conditions, 10% of the glucose metabolized goes through the PPP (Maugeri and Cazzulo 2004). Similar assays were performed in two strains of *L. mexicana* showing that 5.5-5.8% of the glucose consumed is metabolized by the PPP (Maugeri et al. 2003). Atamna et al. (1994) showed that the addition of a permeant scavenger of NADPH, such as methylene blue, increases glucose flux through the PPP by rising the NADP/NADPH ratio, and thus releasing the inhibition of the first enzyme of the pathway, G6PDH, by the reduced coenzyme. This was indeed the case in *T. cruzi*, since in the presence of 0.2 mM methylene blue the glucose flux through the PPP doubled, from 10 to 20% of the total glucose utilization (Maugeri and Cazzulo 2004). A similar situation was observed for promastigotes of both strains of *L. mexicana* tested (Maugeri et al. 2003). These results clearly demonstrate the functionality of the PPP, or

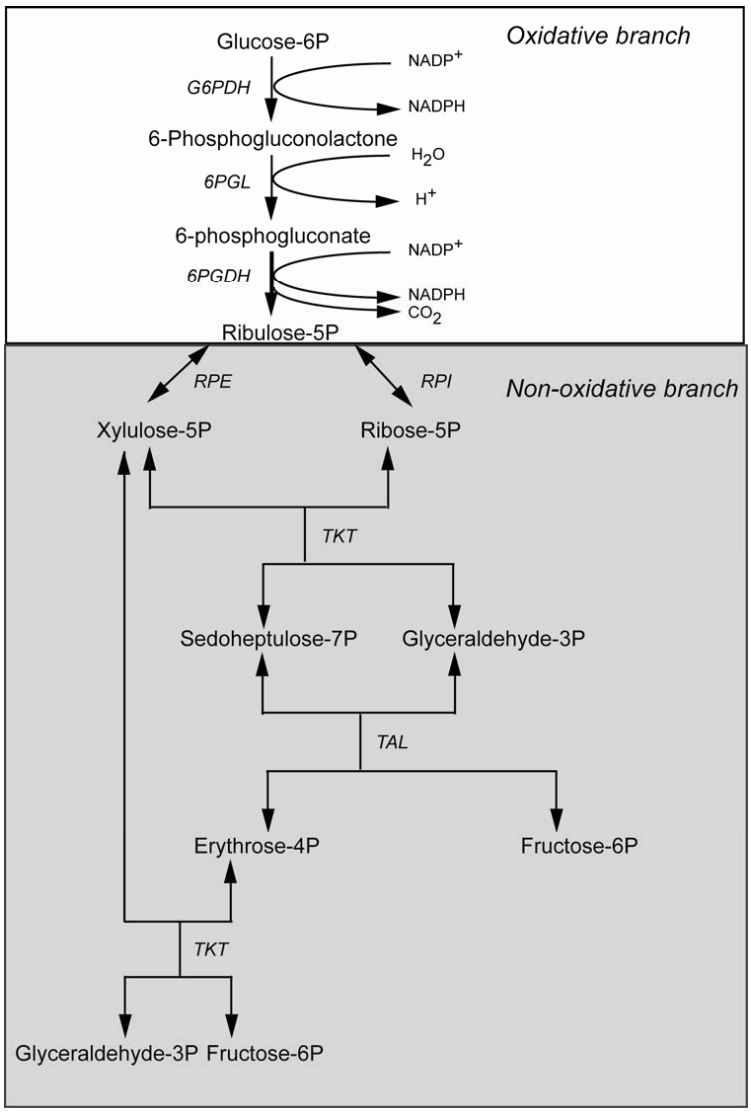

Fig. 1 – Scheme of the pentose phosphate pathway. The enzymes involved are glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconolactonase (6-PGL), 6-phosphogluconate dehydrogenase (6PGDH), ribose 5-phosphate isomerase (RPI), ribulose 5-phosphate epimerase (RPE), transaldolase (TAL) and transketolase (TKT).

at least of its oxidative branch, in the insect stages of both organisms, and suggest that G6PDH activity is also regulated by the NADP/NADPH ratio. In *T. cruzi* the production of ribose 5-phosphate from glucose through the action of the PPP has not been studied but in promastigotes from *L. mexicana* about 11% of the taken glucose was incorporated into nucleic acids (Maugeri et al. 2003).

All seven enzymes of the PPP are present in the four major stages of the biological cycle of *T. cruzi*, epimastigotes, metacyclic trypomastigotes, amastigotes and bloodstream-like trypomastigotes (Maugeri and Cazzulo 2004). With the only exception of RPE, the other enzymes presented their highest activities in metacyclic trypomastigotes. In *T. brucei*, on the other hand, all the enzymes of the PPP seemed to be present in procyclic

trypomastigotes, but some of them could not be detected in the bloodstream form of the parasite (Cronin et al. 1989). In this organism, three of the seven enzymes of the conventional PPP (G6PDH, TAL, RPE) do not have any glycosomal targeting signals although the whole pathway is expected to be present inside this organelle. In epimastigotes from *T. cruzi* all the enzymes of the PPP seem to present multiple subcellular localizations (Maugeri and Cazzulo 2004); however, the bulk of the enzyme activities were detected in the cytosolic fractions, both in digitonin extraction and in subcellular fractionation experiments. The only exception was the RPE, that seems to be localized in a highly digitonin-accessible subcellular compartment, as described below. The three trypanosomatid genomes code for a sedoheptulose-1,7 bisphosphatase (SBPase), an enzyme typical of the Calvin cycle of photosynthetic organisms that can be found only in the chloroplast of green algae and plants. However, the presence of a functional Calvin cycle in kinetoplastids may be excluded. In the three organisms, the protein sequences presented a peroxisomal targeting signal-1 (PTS_1) in their C-terminal suggesting a possible glycosomal localization. Since the Calvin cycle and the PPP are processes which are similarly organized and share a number of enzymes, it has been postulated that in trypanosomatids the SBPase could be involved in a modified PPP (Hannaert et al. 2003). The possible participation of this enzyme in the PPP of *T. cruzi* has not been evaluated until present.

THE ENZYMES OF THE OXIDATIVE BRANCH

GLUCOSE 6-PHOSPHATE DEHYDROGENASE

In *T. cruzi* CL Brener clone, G6PDH is encoded by several genes located in three of the parasite chromosomes (Igoillo-Esteve and Cazzulo 2006). We identified two pseudo-genes and several others that could be clustered in three groups. Those genes were 98% identical in their coding region, but differed considerably in the sequences upstream and downstream of their ORFs. Most of the amino acid changes predicted were conservative, and none involved residues important for catalysis. Sequence comparisons showed that these *T. cruzi* genes had 69% and 64% identity, respectively, with their *T. brucei* and *L. major* counterparts (Fig. 2). The *T. cruzi* and *T. brucei* G6PDH genes presented two candidate start codons, 111 bp apart, while in *L. major* a unique ATG codon, equivalent to the first one observed in *T. cruzi* and *T. brucei*, was present. If the *T. cruzi* G6PDH is translated from the first start codon, a polypeptide chain of 518 amino acid residues is obtained in which the 37 first residues constitute an N-terminal extension compared to the human enzyme. We expressed the *T. cruzi* G6PDH in both, its long and short forms (Igoillo-Esteve and Cazzulo 2006) and compared both recombinant enzymes with the native one. On the other hand, the *T. brucei* G6PDH was only expressed in its short form, starting from the second Met residue (Duffieux et al. 2000). Due to the low levels of expression of the native G6PDH in epimastigotes from *T. cruzi,* added to the instability of the enzyme, we were unable to purify it to homogeneity by standard procedures. A particular feature of the native enzyme was the loss of enzymatic activity in the presence of reducing agents, like β-mercaptoethanol (β-ME) or dithiothreitol (DTT). When the effects of β-ME, as well as those of reduced glutathione (GSH) and DTT were tested on both purified recombinant enzyme forms, we found that the long G6PDH was inhibited by the three reagents, the more prominent effect being obtained when the enzyme was incubated in the presence of DTT (90% inhibition after 15 min incubation with the reducing agent, at 25 mM). Similar but less striking effects were observed while incubating the enzyme with the same concentration of GSH or $\tilde{\beta}$ME (30% and 50% inhibition respectively, after 15 min incubation). Conversely, the short enzyme was activated (up to 80% by 15 mM GSH after the same incubation time). The inhibition of the long G6PDH by reducing agents correlates with the presence of two additional Cys residues in the N-terminal extension of the protein, also observed in redox-regulated G6PDHs from plant chloroplasts and cyanobacteria (Wenderoth et al. 1997). The similar extension predicted from the *L. major* gene does not contain these Cys residues and in good agreement, assays performed with the native enzyme showed that it was not inhibited by DTT (Igoillo-Esteve and Cazzulo 2006). These results suggested that the *T. cruzi* G6PDH, at variance with most G6PDHs from other organisms, might be regulated by the redox state of the cell, as in the case of the chloroplast and cyanobacterial enzymes.

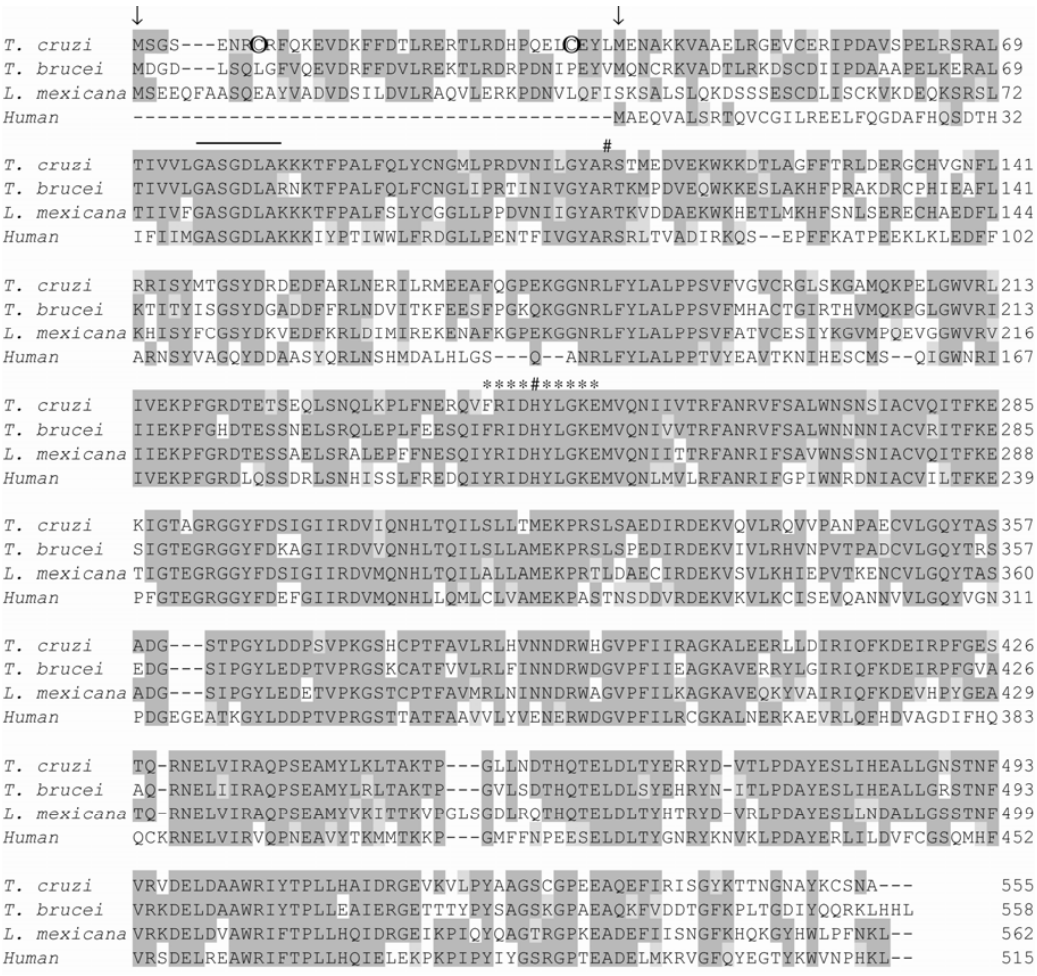

Fig. 2 – Alignment of the deduced amino acid sequences of different glucose 6-phosphate dehydrogenases. The sequences corresponding to the *T. cruzi* G6PDH (DQ408239) with the highest identity to its *T. brucei* counterpart, the *T. brucei* G6PDH (CAC07816), *L. mexicana* G6PDH (AAO37825) and human G6PDH (NP_000393) were aligned using the Clustal program. The first and second methionine in the *T. cruzi* and *T. brucei* sequences are marked with arrows. The two cysteine residues belonging to the *T. cruzi* N-terminal extension are circled. The cofactor binding site is overlined, and the sequence corresponding to the G6PDH signature, that is part of the substrate binding site, is marked with asterisks. Key residues involved in binding the substrate and cofactor are marked with #.

By Western blot analysis performed using an antiserum developed against the N-terminal polypeptide of the long G6PDH, absent in the short form of the enzyme, we could demonstrate that the former is expressed in the four main life stages of *T. cruzi* CL Brener clone. In good agreement, the apparent Km for the substrate, G6P, was similar for the long recombinant enzyme and the native one.

Inhibition kinetics by NADPH performed using the purified short and long *T. cruzi* G6PDHs showed that the latter was 4-fold more sensitive than the short recombinant enzyme to inhibition by the reduced cofactor (Igoillo-Esteve and Cazzulo 2006). These results suggested that, as has been proposed for the G6PDH from *Anacystis nidulans* (Cséke et al. 1981), the activity of the long *T. cruzi* G6PDH may be regulated by the NADP/NADPH pair. We hypothesize that under normal conditions, the enzyme is maintained in its reduced less-active form by NADPH, and under oxidative stress, in which the level of the reduced cofactor drops and other

molecules like H_2O_2 or dehydroascorbate are present, the enzyme is oxidized attaining its more active form. This process would constitute a fast response to increase the NADPH level to counteract the oxidative stress.

The relevance of the G6PDH in the defense mechanisms of *T. cruzi* against oxidative stress was demonstrated by incubating metacyclic trypomastigotes in the presence of different concentrations of H_2O_2. These experiments showed that after 6 hr incubation in the presence of the oxidizing agent at 70 μM, there was a 46-fold increase in G6PDH specific activity, together with an important increase in the protein levels, determined by Western blots. On the other hand, the same experiment performed in epimastigotes, revealed a deleterious effect on the G6PGH since a time-dependent decrease in its specific activity and protein amount was observed (Igoillo-Esteve and Cazzulo 2006).

These experiments showed that in metacyclic trypomastigotes, a form of the parasite naturally exposed to ROS, the G6PDH is strongly induced by oxidative stress. Considering that *T. cruzi* is more sensitive to ROS than mammalian cells, the G6PDH can be considered a suitable target for chemotherapy. Further studies will be required to develop suitable inhibitors, which might become lead compounds for the design of new drugs.

Preliminary studies on the subcellular localization of the G6PDH by immunofluorescence suggest that, in addition to its main cytosolic localization, the enzyme would be also present in glycosomes in amastigotes, trypomastigotes and metacyclic trypomastigotes of *T. cruzi* despite the apparent lack of a PTS_1 or a PTS_2 signal. These experiments, together with G6PDH-GFP fusion analysis, also suggested that the enzyme is probably associated to the Golgi complex in epimastigotes and metacyclic trypomastigotes (Igoillo-Esteve 2005). This localization is not completely unusual since it has been previously suggested for the G6PDH of rabbit intestine cells (Ishibashi et al. 1999). Nevertheless, its biological relevance is not very clear until now; we suggest that in *T. cruzi* a G6PDH fraction associated with the Golgi complex would be indirectly protecting the lipid peroxidation in the organelle membranes via the microsomal Tc-GPXII (Wilkinson et al. 2002b). The G6PDH from procyclic and bloodstream trypomastigotes from *T. brucei* has a dual subcellular localization, cytosolic and glycosomal (Heise and Opperdoes 1999), despite the apparent lack of a PTS signal (Duffieux et al. 2000).

6-PHOSPHOGLUCONOLACTONASE

Studies still in progress show that the *T. cruzi* 6-PGL gene is present in the CL Brener clone as a single copy per haploid genome. A comparison of its sequence with that of the enzyme from *T. brucei* (Fig. 3) indicates that they are 62% identical, and both predict a possible PTS_1 glycosomal targeting signal, placed at the C-terminus in the case of *T. brucei* and internal in the *T. cruzi* 6-PGL (P. Beluardi and J.J. Cazzulo, unpublished results, Duffieux et al. 2000). The *T. cruzi* 6-PGL behaves as a monomeric enzyme, with a molecular mass of 29 kDa. 6-PGL has been recently crystallized from *T. brucei*, and its structure determined by X-ray diffraction. Although the crystals suggest that the enzyme might be a dimer, the authors concluded, by other evidence, that it is most probably a monomer (Delarue et al. 2007). Both trypanosomatid enzymes have been expressed as soluble active recombinant proteins in *E. coli*, but the *T. brucei* enzyme was not characterized, due to the difficulties in the enzyme assay. In fact, the substrate, 6-phosphogluconolactone, must be generated *in situ* by the action of G6PDH on G6P, and undergoes spontaneous hydrolysis, which must be subtracted to get the actual initial reaction velocity. Despite these limitations, we were able to obtain an approximate Km value of 50 μM, close to one of the values reported for the rat liver enzyme (80 μM, Schofield and Sols 1976). The *T. cruzi* 6-PGL seems to be distributed between the cytosol and the glycosome, as reported for the *T. brucei* enzyme.

6-PHOSPHOGLUCONATE DEHYDROGENASE

The 6PGDH from *T. cruzi* is encoded by a single copy gene per haploid genome in the CL Brener clone. The protein has 78.6% identity with its *T. brucei* counterpart (Igoillo-Esteve and Cazzulo 2004). Both, the native and the recombinant 6PGDHs from *T. cruzi* proved to be unstable, and the native enzyme could only be partially purified from parasite extracts. Such instability has not been reported for the *T. brucei* enzyme. Structural analysis of the *T. brucei* 6PGDH dimer suggested that 5 salt bridges between subunits are essential for the stabilization of the active dimeric enzyme (Phillips et al. 1998).

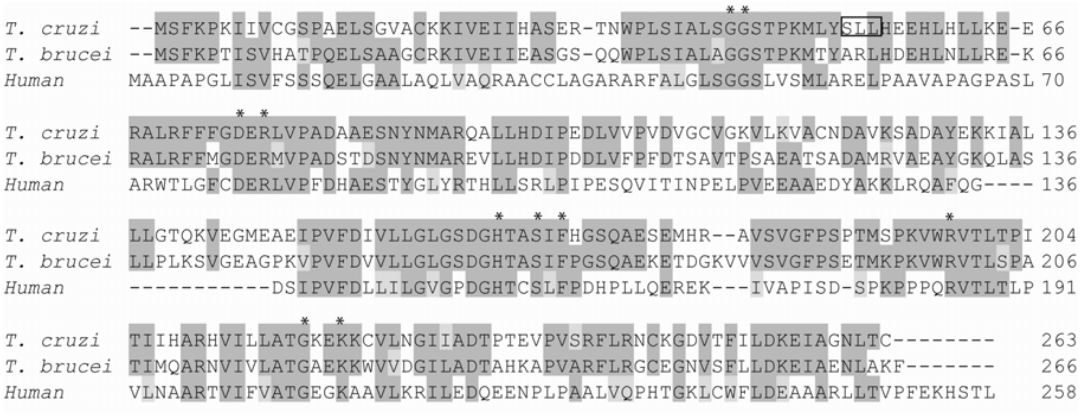

Fig. 3 – Alignment of the deduced amino acid sequences of different 6-phosphogluconolactonases. The accession numbers for the sequences aligned using the Clustal program are the following: *T. cruzi* EU077554, *T. brucei* AJ249255, Human AJ243972. Boxes shaded in dark grey show correspond to identical residues. The asterisks show the residues strictly conserved in the 6PGL family. Previous studies (Delarue et al. 2007) suggest that R77, F170, and R198 in the *T. cruzi* sequence could constitute the 6PGL signature. The grey box shows a putative internal PTS in the *T. cruzi* sequence.

A comparison of the *T. cruzi* and *T. brucei* 6PGDH sequences (Fig. 4) showed that in the former two residues which are involved in the formation of two out of the five mentioned salt bridges are lacking. Considering that this might be the reason for the instability of the enzyme from *T. cruzi*, we performed site-directed mutagenesis, leading to a double mutant protein which, according to computational modeling, would have the five bridges. This mutant proved to be considerably more stable than the wild type enzyme and non mutated recombinant 6PGDH, therefore allowing its kinetic study. By gel filtration analysis we could determine that the double mutant enzyme remained as a dimer under conditions which led to very significant dissociation of the non mutated one (Igoillo-Esteve and Cazzulo 2004).

Hanau et al. (1996) performed detailed kinetic analysis of the *T. brucei* 6PGDH; the enzyme presented unusually low Km values, 3.5 μM for the substrate and 1.5 μM for the coenzyme. In contrast we found that for the *T. cruzi* enzyme, the Km for 6-phosphogluconate was similar to that of the human 6PGDH, but the Km for NADP (5.9 μM), although 4-fold higher than that for the *T. brucei* enzyme, was still 5-fold lower than the one reported for the human one (30 μM) (Igoillo-Esteve and Cazzulo 2004).

It is noteworthy that deletion of 6PGDH is lethal for all cells studied, since accumulation of 6-phosphogluconate inhibits phosphoglucose isomerase, thus blocking glycolysis. Therefore, specific inhibitors for the parasite's 6PGDH could become good lead compounds for the development of new drugs. Dardonville and co-workers performed inhibition studies on the *T. brucei* 6PGDH utilizing several substrate and cofactor analogues (Dardonville et al. 2003), as well as high-energy intermediates and transition-state analogues (Dardonville et al. 2004). A few of these compounds were tested on the purified recombinant double-mutant *T. cruzi* 6PGDH and the best result, K_i 0.16 μM, was obtained for 4-phospho-D-erythronate, an analogue of the enediol-reaction intermediate (M. Igoillo-Esteve and J.J. Cazzulo, unpublished results). This compound also showed a selective inhibition of 6PGDH from *T. brucei* over the sheep liver enzyme, (K_i (*T. brucei*) 0.13 μM and K_i (sheep) 10.7 μM) (Pasti et al. 2003). Additionally Dardonville et al. (2003) have reported that some inhibitors developed for the *T. brucei* enzyme were toxic to *T. cruzi* amastigotes at concentrations lower than 10 μM. These results indicate that it would be possible to extrapolate to *T. cruzi* the utilization of some of the compounds designed to inhibit the *T. brucei* 6PGDH that nowadays are being improved to increase their cellular permeability to reach their target *in situ*.

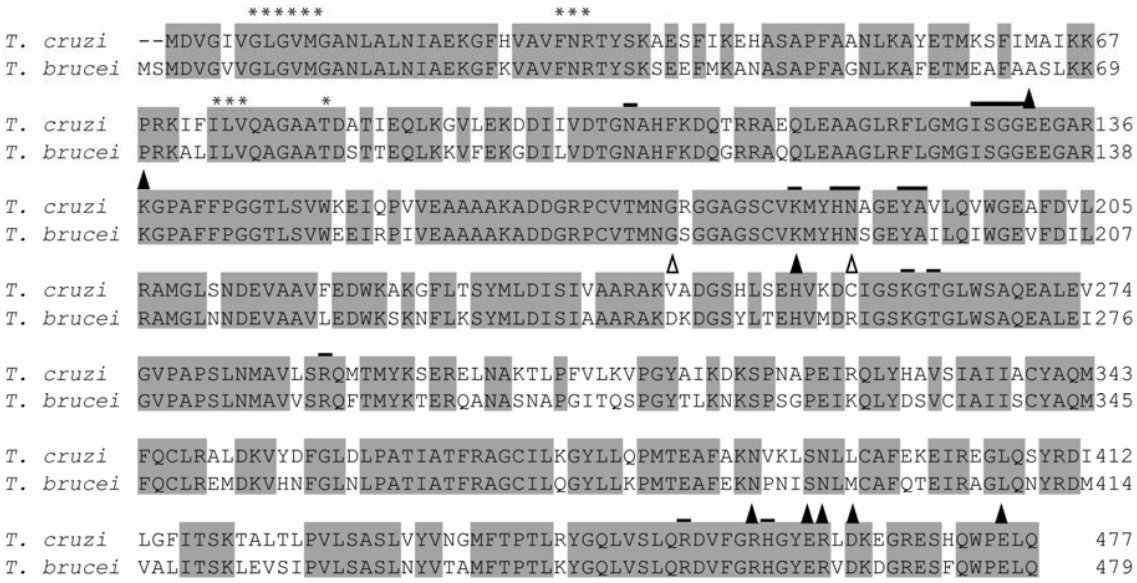

Fig. 4 – Alignment of the deduced amino acid sequences of different 6-phosphogluconate dehydrogenases. The 6-PGDHs from *T. cruzi* (AY300924) and *T. brucei* (P31072) were aligned using the Clustal program. The asterisks indicate the residues comprising the coenzyme binding site, and those involved in substrate binding are overlined. The triangles indicate the residues involved in the formation of salt bridges in the *T. brucei* enzyme; empty triangles indicate those absent in the *T. cruzi* enzyme.

The subcellular localization of the *T. cruzi* 6PGDH was analyzed by immunofluorescence. These experiments revealed that in addition to the main cytosolic localization, a small fraction of the enzyme would be present inside the glycosome, at least in epimastigotes and cell culture-trypomastigotes (Igoillo-Esteve 2005). This localization is in agreement with a possible internal PTS_1 – like sequence (SHL) localized in a predicted exposed loop of the protein (Fig. 4).

THE ENZYMES OF THE NON-OXIDATIVE, OR SUGAR INTERCONVERSION, BRANCH

RIBOSE 5-PHOSPHATE ISOMERASE

The haploid genome of the CL Brener clone of *T. cruzi* contains one gene coding for a Type B RPI. However, genes encoding Type A RPIs, most frequent in eukaryotes, seem to be absent. So far, RPI B eukaryotic genes have been detected only in *Giardia lamblia*, *Entamoeba histolytica*, some fungi and the insect *Anopheles gambiae*. The deduced amino acid sequence of the *T. cruzi* RPI B does not predict a PTS_1 or PTS_2 glycosomal targeting signal (Fig. 5). The RPI from *T. cruzi* is another example of an enzyme which is absent in upper eukaryotic genomes (Stern et al. 2007), since Type A and B RPIs are totally unrelated. A recombinant enzyme was expressed as an active dimeric protein, able to catalyze the reversible isomerization of R5P to Ru5P, with Km values of 4 mM and 1.4 mM, respectively. We were able to study the reaction mechanism of this Type B RPI using several approaches, including site-directed mutagenesis. The competitive inhibition of the enzyme by 4-phospho D-erythronohydroxamic acid showed that this RPI acts *via* a mechanism involving the formation of a 1,2-cis enediol. The reaction in the direction R5P to Ru5P, but not the reverse reaction, must involve the opening of the ribose furanose ring. Using site-directed mutagenesis, based on the modeling of the enzyme from *T. cruzi* using the *E. coli* RPI B structure, we were able to show that His^{102}, but not His^{138}, was essential for ring opening. Moreover, we were able to confirm the essential role of Cys^{69} in catalysis. These results not only gave interesting information on the reaction mechanism of Type B RPIs, but suggest that, considering its absence in upper eukaryotes, this enzyme might become an important target for the chemotherapy of Chagas disease (Stern et al. 2007).

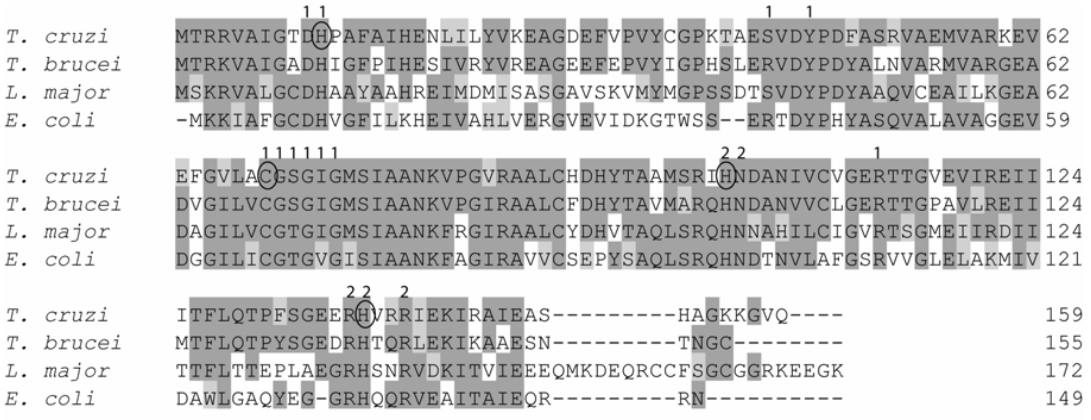

Fig. 5 – Alignment of the deduced amino acid sequences of different ribose 5-phosphate isomerases. The TcRPIB (gi: 110984574) is aligned with those of *T. brucei* (gi:70834348), *L. major* (gi:68127548) and *E. coli* (gi: 85676843 W3110). Mutagenizated aminoacid residues are circled. Residues that lie within the RPI active-site pocket are annotated with 1 for one subunit and with 2 for the second.

Ribulose 5-phosphate epimerase

RPE catalyses the interconversion of two phosphorylated pentoses, R5P and xylulose 5-phosphate (X5P). The genome of the CL Brener clone of *T. cruzi* contains two genes encoding RPEs. One of them predicts a PTS_1 glycosomal targeting signal (SHL) at the C terminus (Fig. 6). Digitonin extraction studies showed that RPE activity is extracted from the epimastigotes by very low concentrations of the detergent (about 0.3 mg/ml), lower than those required to extract the pyruvate kinase used as cytosolic marker (Maugeri and Cazzulo 2004). Other enzymes, like some quinone oxidoreductases (QOR), have a similar extraction pattern (J.J.B. Cannata and D. Maugeri, unpublished results, Fig. 7), suggesting the possibility of the existence of a new, highly digitonin-accessible, subcellular compartment. It is noteworthy that, in cellular fractionation experiments, almost 50% of the RPE activity behaves as particulate, with latency. Both RPE forms have been cloned and expressed as active proteins in *E. coli*, and are being characterized at present, in order to define their subcellular localization and possible functions (A.L. Stern and J. J. Cazzulo, unpublished results).

Transaldolase

Transaldolase (D-sedoheptulose-7-phosphate: D-glyceraldehyde - 3 - phosphate - dihydroxyacetone transferase, EC 2.2.1.2, TAL), transfers a dihydroxyacetone unit from F6P to erythrose 4-phosphate (E4P), leading to the synthesis of sedoheptulose 7-phosphate and Gly3P. A Schiff base involving a Lys residue present in the active site and dihydroxyacetone is essential in the reaction mechanism. Different lines of evidence suggest that TAL has an essential regulatory role in the non-oxidative branch of the PPP, at least in higher eukaryotes (Reitzer et al. 1980, Banki et al. 1996, Heinrich et al. 1976).

We have cloned the gene encoding TAL from the CL Brener clone of *T. cruzi*, which is present as a single copy per haploid genome (Fig. 8), and expressed the recombinant protein in *E. coli* as an active enzyme, with apparent Km values of 1 mM for F6P and 0.02 mM for E4P. Preliminary results suggest the presence of four TAL isoforms in epimastigotes. At this moment we are working in the purification of the native isoforms, in an attempt to elucidate their role in the parasite metabolism (A.L. Stern and J.J. Cazzulo, unpublished results).

Transketolase

The genome of the CL Brener clone contains a gene encoding TKT, which seems to be present as a single copy per haploid genome. The gene, which predicts 67% identity with the recently characterized TKT from *L. mexicana* (Veitch et al. 2004, Fig. 9), was cloned and the protein expressed as a fusion with a polyHis tag in the N-terminus, in *E. coli* BL21 Codon Plus. The recombinant protein was purified by IMAC, and showed to be

```
                                                                                  ***** *
T._cruzi_1 -----MFDHQDRGTWLSADRPD--TLRPIIAPSILASDFARLLDECKDVLSVEGGASEWLHVDVMDGH 61
T._cruzi_2 ---MRSFDHQDKSTFLSGRRKK--PLRPIISPSIMVADPTQLLLEALHVLSPEGGAVEWLHVDVIDGH 63
T._brucei  -----MVHRQDGAVVHCSRETG--RLSAIIAPSILASDFAQLLSECQSVLSPEGGAADWLHVDVMDGH 61
L._major   MTEFNRYNHEDKSTWLNAVEKPQAPLEAIIDPSILAADFCKLGDEVASVVSPAGGAVEWIHVDVMDGH 68
Human      ----------------------MASGCKIGPSILNSDLANLGAECLRMLDSG---ADYLHLDVMDGH 42
           ** * *
T._cruzi_1 LVPNISIGMCVVEALRKHL-QHTFLDVHCMVSHPDRWVDEMAKAGASQMTFHVEAAECPKAVARHIRA 128
T._cruzi_2 FAPNMSFCSDTVAGLRRHL-PHVFLDVHLMVAEPEKWVRPFADAGASQFTFHIEATQEPVALASKIRE 130
T._brucei  FVPNISIGMCVVQSLRSHL-QDVFLDVHCMVSDPDRWVEEMAKAGATQMTFHVEAVADPKAVARHIRA 128
L._major   MVPNISIGPGVVSSLRARF-PNVFLDVHCMVSDPKQWVPSIAKAGGSGYTFHIEATNDSKGVAEHIRA 135
Human      FVPNITFGHPVVESLRKQLGQDPFFDMHMMVSKPEQWVKPMAVAGANQYTFHLEATENPGALIKDIRE 110
                                        * **** *   ** *     **
T._cruzi_1 AGMQYGVALKPKT-LASAVTELIEEKLVDMVLVMTVEPGFGGQSFMHDMMPKVAELRRAYPHLNIQVD 195
T._cruzi_2 AGMQVGVAISPRT-AIDGLIPLIENGHLDMVLVMTVEPGFAGQKFLSEPLAKVRQLRGRYPHLNIQVD 197
T._brucei  AGMLCGVALKPKT-PVSSVLNLVEEQLIDMVLVMTVEPGFGGQSFMQDMMPKVEELRARFPCLNIQVD 195
L._major   HGMQVGVAVKPATNLTQELRDLIEGHYVDQVLVMTVEPGFGGQSFIQERLSFISELRRNYPHLNIGVD 203
Human      NGMKVGLAIKPGT---SVEYLAPWANQIDMALVMTVEPGFGGQKFMEDMMPKVHWLRTQFPSLDIEVD 175

T._cruzi_1 GGLGEKTIDAAAEAGANIIVAGTSIFKAAVRKQATETMRNAVKKSLTPK---------- 244
T._cruzi_2 GSINLVTVDEAALAGANCLVPGQAVFKAKNRQENVIHLRRVVHHHLKSHL---------- 247
T._brucei  GGIGPGTIDAPARAGANVIVAGTSIFRADSRKEATESMRRVVQGYLGSTTS-------- 246
L._major   GGIGPSTAVQAADAGANILIAGTSVFRAGDRKAAIEELRASAKSGICKNSIQRALPGMR 262
Human      GGVGPDTVHKCAEAGANMIVSGSAIMRSEDPRSVINLLRNVCSEAAQKRSLDR------ 228
```

Fig. 6 – Alignment of the deduced amino acid sequences of different ribulose 5-phosphate epimerases. The two epimerases from *T. cruzi* (TcRPE1, EU075265 and TcRPE2, EU075366) are aligned with those of *T. brucei* (XP_823426), *L. major* (CAJ06401) and human (NP_954699). Tc Rpe2 presents a PTS_1 (SHL) in the C-terminus. The asterisks indicate the residues conserved in the RPE signature.

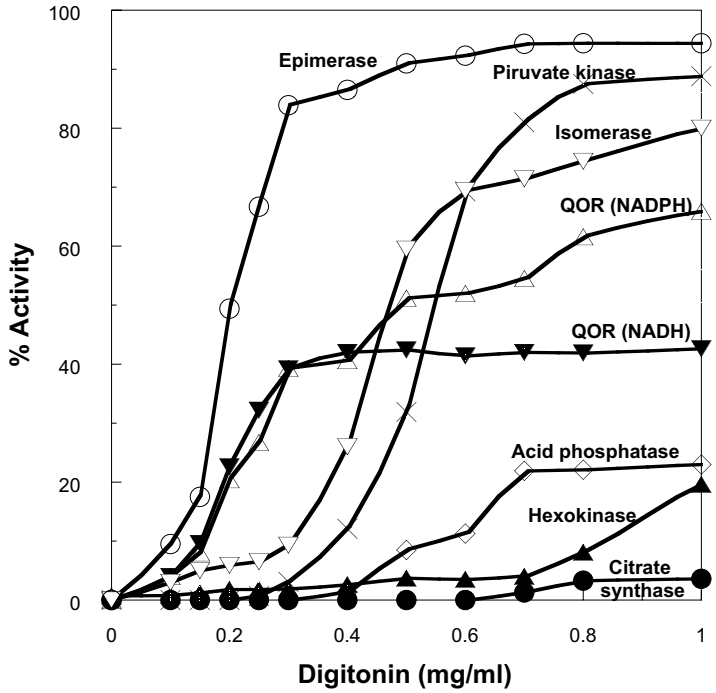

Fig. 7 – Behaviour of ribulose 5-phosphate epimerase activity upon digitonin extraction of *T. cruzi* epimastigotes. The experiment was perfomed as described by Maugeri and Cazzulo (2004).

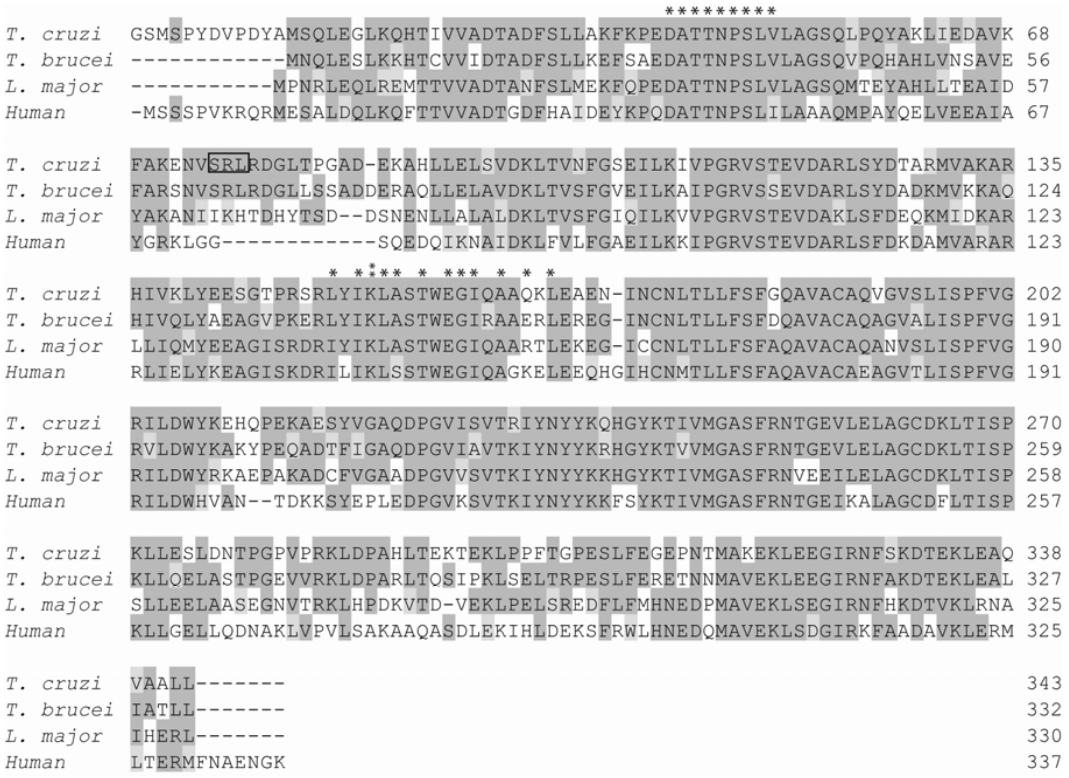

Fig. 8 – Alignment of the deduced amino acid sequences of different transaldolases. The *T. cruzi* TAL (EU075264) is aligned with those of *T. brucei* (XP_847390), *L. major* (CAJ03645) and human (NP_006746). A putative PTS_1 (SRL) is boxed. The asterisks indicate the residues conserved in the TAL signature.

a dimer (146 kDa) acting on R5P, E4P and X5P with apparent Km values of 1.34 mM, 0.1 mM and 0.07 mM, respectively, at the optimum pH value of 8.0. The enzyme is expressed in the four main stages of *T. cruzi*, with an apparent subunit molecular mass of 75 kDa, as determined by Western blot (P. Beluardi and J.J. Cazzulo, unpublished results). The *L. mexicana* TKT has been recently characterized, and its 3-D structure has been determined (Veitch et al. 2004). It has been demonstrated that the enzyme, that bears a C-terminal PTS_1, has a dual localization, cytosolic and glycosomal, in *Leishmania* promastigotes. Possibly the *T. cruzi* TKT shares the same location since it also presents a PTS_1 signal in its C-terminal region (Fig. 9) but this point still needs to be confirmed.

CONCLUSIONS

The PPP has been shown to be functional in *T. cruzi*, and the seven enzymes of the pathway have been cloned, expressed and are being fully characterized. The biochemical evidence obtained so far suggest that the oxidative branch of the PPP is essential for the protection of the parasite against oxidative stress. In addition, one of the enzymes of the non-oxidative branch is of prokaryotic type, and has no counterpart in the higher eukaryotic genomes sequenced until now.

Apart from its main cytosolic localization, the PPP is expected to be present, at least partially, inside the glycosomes, in order to produce the NADPH and R5P required for other enzymatic pathways present inside the organelle. R5P will be converted into 5-phosphoribosyl-1-pyrophosphate that will serve in purine and pyrimidine biosyntheses, processes that occurred inside the glycosomes. On the other hand, NADPH would be involved in the defense against reactive oxygen species (ROS) since, as mentioned above, Tc-GPXI is located in *T. cruzi* glycosomes. Moreover, indications have been obtained for the presence of trypanothione reductase not only in

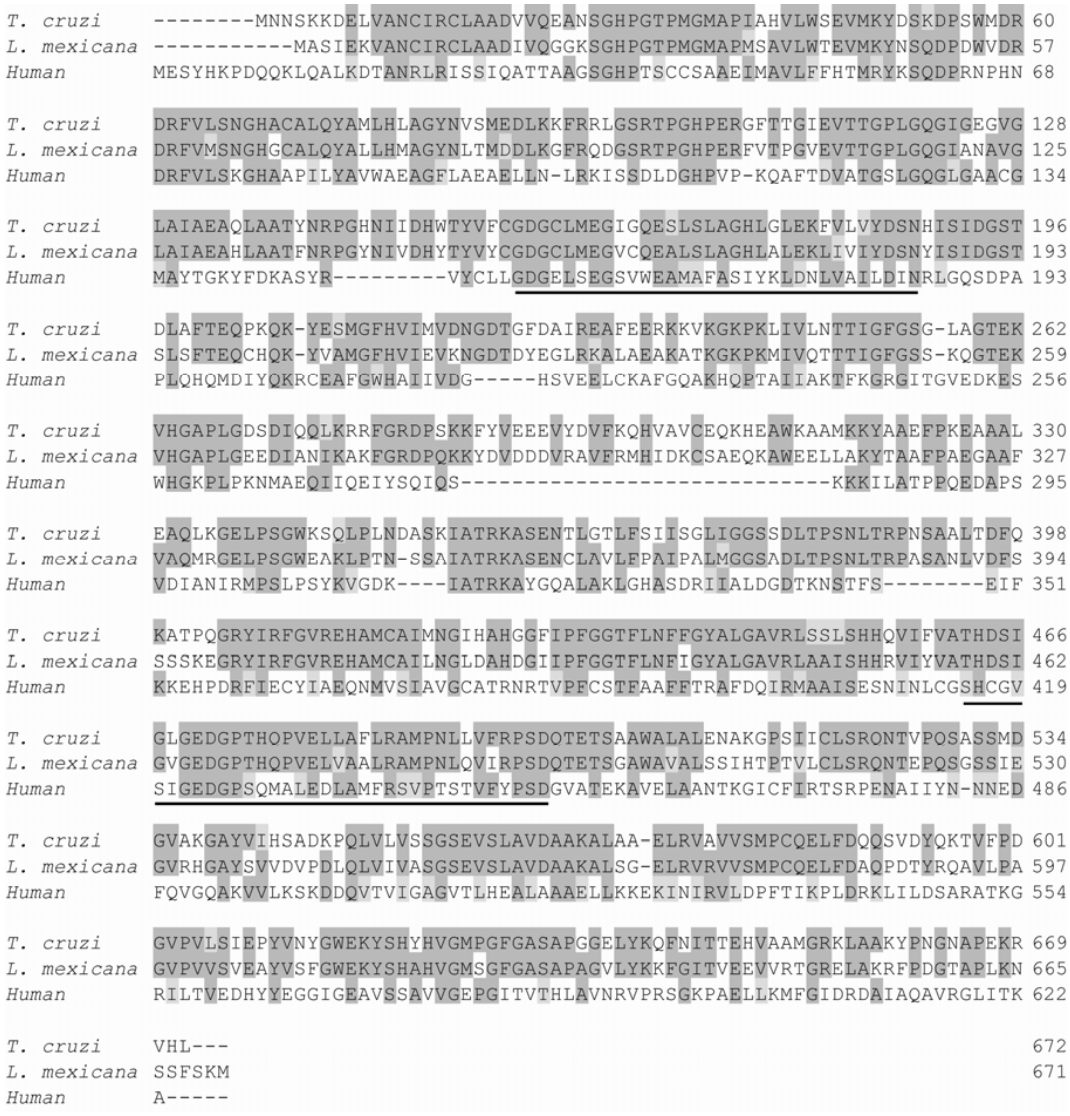

Fig. 9 – Alignment of the deduced amino acid sequences of different transketolases. The accession numbers for the sequences aligned using the Clustal program are the following: *T. cruzi* EU077555, *L. mexicana* Aj427448, human P29401. Boxes shaded in dark grey show correspond to identical residues. The conserved TKT and ThDP boxes, which are involved in cofactor and substrate union, are underlined.

the cytosol but also in the glycosomes of *T. cruzi* and *T. brucei*. These defense systems are, via trypanothione reductase, ultimately dependent on NADPH produced through the PPP (Hannaert et al. 2003). Although we have demonstrated that at least the oxidative branch of the pathway might be present inside the organelle in some developmental stages of *T. cruzi*, further experiments are required in order to confirm or discard the glycosomal localization of the whole PPP in this parasite.

Finally, more studies, involving inhibitor kinetics and knock-out experiments, will be required to validate the PPP as a suitable target for the development of new drugs for the treatment of Chagas Disease.

ACKNOWLEDGMENTS

The work at the authors' laboratory mentioned in this review was done aided by grant PICT 2000-01849 from ANPCyT, SECYT, Argentina and SB06/27 from UNSAM. MI-E, DM, ALS and PB were research fel-

lows from ANPCyT, UNSAM, Fundación Repsol/YPF and CONICET, respectively, and JJC is a member of the Research Career of CONICET.

RESUMO

Trypanosoma cruzi é altamente sensível ao estresse oxidativo causado por espécies reativas do oxigênio. Tripanotiona, o principal protetor do parasita contra o estresse oxidativo, é mantido reduzido pela tripanotiona redutase, pela presença de NADPH; a principal fonte da coenzima reduzida parece ser a via da pentose fosfato. As sete enzimas dessa via estão presentes nos quatro principais estágios do ciclo biológico do parasita; nós clonamos e expressamos as enzimas em *Escherichia coli* como proteínas ativas. Glucose 6-fosfato desidrogenase, que controla o fluxo da glucose da via em resposta à relação NADP/NADPH, é codificada por um número de genes por genoma haplóide e é induzida até 46-vezes por peróxido de hidrogênio em trypomastigotas metacíclicos. Os genes que codificam 6-fosfogluconolactonase, 6-fosfogluconato desidrogenase, transaldolase e transcetolase estão presentes no clone CL Brener como cópia única por genoma haplóide. 6-fosfogluconato desidrogenase é muito instável, mas foi estabilizada introduzindo duas pontes salinas por mutagênese sítio-dirigida. A Ribose-5-fosfato isomerase pertence ao Tipo B; genes que codificam enzimas Tipo A, presentes em mamíferos estão ausentes. A Ribulose-5-fosfato epimerase é codificada por dois genes. As enzimas da via têm um componente citosólico principal, embora várias delas tenham uma localização glicosomal secundária e também, localizações em menor número em outras organelas.

Palavras-chave: *Trypanosoma cruzi*, doença de Chagas, via das pentoses fosfato, estresse oxidativo, produção de NADPH.

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