

# Ribose 5-phosphate isomerase type B from *Trypanosoma cruzi*: kinetic properties and site-directed mutagenesis reveal information about the reaction mechanism

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*Trypanosoma cruzi*, the human parasite that causes Chagas disease, contains a functional pentose phosphate pathway, probably essential for protection against oxidative stress and also for R5P (ribose 5-phosphate) production for nucleotide synthesis. The haploid genome of the CL Brener clone of the parasite contains one gene coding for a Type B Rpi (ribose 5-phosphate isomerase), but genes encoding Type A Rpis, most frequent in eukaryotes, seem to be absent. The RpiB enzyme was expressed in *Escherichia coli* as a poly-His tagged active dimeric protein, which catalyses the reversible isomerization of R5P to Ru5P (ribulose 5-phosphate) with  $K_m$  values of 4 mM (R5P) and 1.4 mM (Ru5P).

4-Phospho-D-erythronohydroxamic acid, an analogue to the reaction intermediate when the Rpi acts via a mechanism involving the formation of a 1,2-*cis*-enediol, inhibited the enzyme competi-

tively, with an  $IC_{50}$  value of 0.7 mM and a  $K_i$  of 1.2 mM. Site-directed mutagenesis allowed the demonstration of a role for His<sup>102</sup>, but not for His<sup>138</sup>, in the opening of the ribose furanose ring. A major role in catalysis was confirmed for Cys<sup>69</sup>, since the C69A mutant was inactive in both forward and reverse directions of the reaction. The present paper contributes to the knowledge of the mechanism of the Rpi reaction; in addition, the absence of RpiBs in the genomes of higher animals makes this enzyme a possible target for chemotherapy of Chagas disease.

**Key words:** Chagas disease, pentose phosphate pathway, reaction mechanism, ribose 5-phosphate isomerase (Rpi), *Trypanosoma cruzi*, Type B ribose 5-phosphate isomerase (RpiB).

## INTRODUCTION

*Trypanosoma cruzi*, the parasitic protozoan which causes American trypanosomiasis (also known as Chagas disease) has a functional PPP (pentose phosphate pathway). A crucial role for the PPP in the protection of trypanosomatids against oxidative stress and in the provision of nucleotide precursors has been suggested [1,2]. All seven enzymes of the PPP are present in the four major stages in the biological cycle of the parasite, namely the epimastigote and the metacyclic trypomastigote present in the insect vector, and the intracellular amastigote and the bloodstream trypomastigote present in the infected mammal [1].

The PPP consists of two branches, the oxidative branch leading from glucose 6-phosphate to Ru5P (ribulose 5-phosphate), with the reduction of two molecules of NADP, and the non-oxidative or sugar interconversion branch, which ultimately leads back to glycolytic intermediates. Rpi (ribose 5-phosphate isomerase; EC 5.3.1.6) is a key enzyme of the PPP which catalyses the reversible aldose-ketose isomerization between R5P (ribose 5-phosphate) and Ru5P. Two completely unrelated enzyme proteins are able to catalyse this reaction [3,4]. In *Escherichia coli*, one of them, RpiA, is a constitutively expressed 23 kDa protein [5,6], whereas the other, RpiB, is a 16 kDa protein, whose expression is regulated by a repressor [7]. Expression of either enzyme allows normal growth of *E. coli*, but the double mutant *rpiA*<sup>-</sup>/*rpiB*<sup>-</sup> is severely impaired

for growth under all experimental conditions tested, showing that the reaction itself is very important for the bacterium [7].

RpiAs are broadly distributed in the three kingdoms of life, including most eukaryotic organisms, fungi and some bacteria. On the other hand, inspection of the protein family data base, Pfam (accession number PF02502) showed that most RpiBs are found in prokaryotic organisms; there are few exceptions in lower eukaryotes, such as *Giardia lamblia*, *Entamoeba histolytica* and some fungi, and in the insect *Anopheles gambiae*.

Looking for Rpi homologues in the *T. cruzi* genome, we found that while the RpiB enzymes seemed to be present in the parasite, RpiA homologues were completely absent. Thus TcRpiB (*T. cruzi* RpiB) turned out to be the only enzyme of the *T. cruzi* PPP which does not have its own counterpart in higher eukaryotes.

As with most sugar phosphate isomerases, both RpiAs and RpiBs seem to act via a *cis*-enediol(ate) intermediary mechanism instead of a hydride transfer mechanism which has been reported to occur for some sugar isomerases such as D-xylose isomerase [8] and L-rhamnose isomerase [9]. Prior to isomerization, it is believed that RpiAs and RpiBs take from solution furanose R5P which represents the majority in solution; however the true substrate for the isomerization step is the far less abundant R5P in the free aldehyde form. Furthermore, R5P isomerization gives the acyclic product Ru5P, which is also the substrate of the enzyme. For these reasons it has been proposed that the enzyme first

Abbreviations used: EcRpiB, *Escherichia coli* ribose 5-phosphate isomerase B; MESNA, sodium 2-mercapto-ethanesulfonate; MtRpiB, *Mycobacterium tuberculosis* ribose 5-phosphate isomerase B; 4PEA, 4-phospho-D-erythronate; 4PEAm, 4-phospho-D-erythronamide; 4PEH, 4-phospho-D-erythronohydroxamic acid; 4PEHz, 4-phospho-D-erythronhydrazide; 5PRH, 5-phospho-D-ribonohydroxamic acid; PPP, pentose phosphate pathway; R5P, ribose 5-phosphate; Rpi, ribose 5-phosphate isomerase; Ru5P, ribulose 5-phosphate; TBS, tris-buffered saline; TcRpiB, *Trypanosoma cruzi* ribose 5-phosphate isomerase B.

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Nucleotide sequence data reported in the present paper are available in the GenBank® database under the accession number DQ782334.

catalyses the opening of the furanose ring of R5P. Structural data on RpiBs from *Mycobacterium tuberculosis* and *E. coli* gave strong insights about the RpiB mechanism and the roles of some amino acid residues were proposed [10–12], but no definitive proof was given.

In the present paper, we describe the overexpression of the TcRpiB and some general properties of the purified recombinant enzyme. Two key amino acid residues, one of them involved in the isomerization step and the other involved in the ring opening step, have been identified by site-directed mutagenesis. The demonstration of the fact that the RpiB is the only Rpi present in the parasite, and has no homologue in higher animals, opens up the possibility of considering it as a new potential target for the chemotherapy of Chagas disease.

## MATERIALS AND METHODS

### Chemicals and reagents

R5P was purchased from Sigma. MESNA (sodium 2-mercaptoethanesulfonate) and Ru5P were purchased from Fluka Chemie GmbH. Oligonucleotide primers were from Gibco, Life Technologies. dNTPs (deoxynucleoside-5'-triphosphates) were from Promega. Restriction endonucleases were from New England Biolabs. *E. coli* strain BL21 codon Plus (DE3) and the QuickChange™ site-directed mutagenesis kit were purchased from Stratagene. Polyclonal antibodies against TcRpiB were obtained in rabbits inoculated with purified recombinant His-tagged TcRpiB.

### Parasites and culture

Epimastigotes of the CL Brener strain of *T. cruzi* were grown in axenic medium, harvested and washed as previously described [13]. Metacyclic trypomastigotes were obtained by spontaneous differentiation of epimastigotes of the CL Brener clone at 28 °C, followed by purification using DEAE-cellulose chromatography [14]. Amastigotes and trypomastigotes were obtained by infection of Vero cell monolayers with trypomastigotes of the CL Brener strain [15]. Trypomastigotes were obtained free of cellular debris by leaving them to swim off the centrifuged pellet by incubating for 1 h at 37 °C [15]. Cell-free extracts were obtained in 50 mM Tris/HCl buffer (pH 7.8) containing 0.5 mM TLCK (tosyl-lysyl-chloromethyl ketone) and 4 mM dithiothreitol, by three cycles of freezing at –20 °C and thawing, followed by sonication in a Branson 450 Sonifier, by three continuous pulses (20 s each) at 60% of maximal power. The suspensions were centrifuged at 20000 g for 10 min.

### Cloning of the Rpi gene

*TcRpiB* was obtained by performing PCR on genomic DNA from *T. cruzi* epimastigotes. Primers were designed according to the sequence data obtained from the *T. cruzi* Genome Project database search. The sequences of the primers were as follows: sense primer 5'-GCTAGCATGACGCGCCGAGTTCG-3' and antisense primer 5'-TGCATTCTGTACATCATTCTCG-3'. PCR conditions were as follows: initial denaturation (5 min at 94 °C), 25 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), elongation (60 s at 72 °C) and a final extension step (10 min at 72 °C). The PCR products were isolated from a 1% agarose gel, purified by the Qiaex II protocol (Qiagen), and cloned into a pGEM-T Easy vector (Promega). Sequencing of the products was performed using an ABI Prism® 377 DNA sequencer (PerkinElmer).

### Expression and purification of poly-His tagged recombinant TcRpiB

The *TcRpiB* gene was excized as a NheI/SalI fragment from the pGEM-T Easy vector, gel purified and subcloned into the NheI and XhoI sites of the pET28a(+) expression vector (Novagen). The resulting construct presented a poly-His tag at the N-terminal and was transformed into *E. coli* BL21 codon Plus (DE3) cells. The recombinant protein was expressed by induction of log-phase cultures (200 ml;  $D_{600} = 0.6$ ) with 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) for 3 h at 37 °C with shaking at 250 rev./min. Bacteria were harvested by centrifugation (5000 g for 30 min at 4 °C), resuspended in 20 ml of 50 mM Tris/HCl (pH 7.6) containing 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF and 0.2 mg/ml lysozyme, and centrifuged (12000 g for 30 min at 4 °C) to obtain the bacterial crude extract. The recombinant enzyme was purified in one step using Ni<sup>2+</sup> resin (ProBond) pre-equilibrated in 50 mM Tris/HCl (pH 7.6) containing 500 mM NaCl. The column was washed sequentially with 50 ml of the equilibration buffer, 50 ml of the equilibration buffer plus 50 mM imidazole and 2 ml of the equilibration buffer plus 100 mM imidazole. TcRpiB was eluted with the equilibration buffer plus 300 mM imidazole. Depending on the assay method to be used with the sample, dialysis was performed against 50 mM Tris/HCl (pH 7.6), 150 mM NaCl containing either 1 mM EDTA and 0.5 mM 2-mercaptoethanol or 5 mM MESNA.

### Molecular mass determination

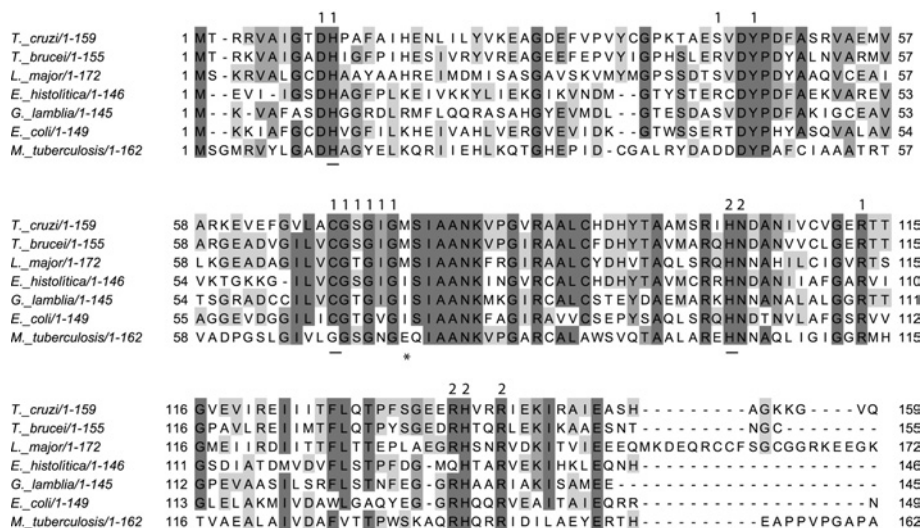
The TcRpiB apparent subunit molecular mass was estimated by SDS/PAGE [16]. The active TcRpiB molecular mass was determined by gel-filtration in a Bio-Sil SEC 250 HPLC column (Bio-rad) eluted with 50 mM Tris/HCl buffer (pH 7.6) containing 150 mM NaCl. Column calibration was performed using bovine thyroglobulin (670 kDa), bovine  $\gamma$ -globulin (158 kDa), chicken ovalbumin (44 kDa) and horse myoglobin (17 kDa) as molecular mass standards.

### Western blot analysis

For Western blots, soluble *T. cruzi* proteins (30  $\mu$ g) were resolved on SDS/PAGE (15% gels), transferred on to a nitrocellulose Hybond ECL® membrane (Amersham Biosciences), blocked in 3% (w/v) non-fat dried skimmed milk in TBS [Tris-buffered saline; 2% glycine and 150 mM NaCl in 50 mM Tris/HCl (pH 7.6)] (blocking buffer) followed by incubation with an anti-TcRpiB antibody (1:500 for 1.5 h). Blots were washed with TBS (2  $\times$  10 min), 0.05% Nonidet P-40 in TBS (1  $\times$  10 min) and TBS (2  $\times$  10 min). Peroxidase-conjugated goat anti-rabbit IgG (Sigma) (1:10000 for 1 h) in blocking buffer was used as the secondary antibody. The membranes were washed again and developed using SuperSignal WestPico Chemiluminescent Substrate (Pierce).

### Enzyme assay

Rpi activity was assayed by three different methods. First, to determine the  $K_m$  for R5P, to test inhibitors and to evaluate the pH effect over the enzymatic activity a direct spectrophotometric method at 290 nm was used [17] in 100 mM Tris/HCl (pH 7.6). Inhibitors were tested in the presence of 3.12 mM R5P.  $K_m$  determination was performed at R5P concentrations in a range between 3.1 and 50 mM. An absorbance of 0.072 at 290 nm was considered for 1 mM Ru5P. Secondly, to determine the  $K_m$  for Ru5P, and to test metals and metal chelators and the effect of iodoacetamide, a modification of Dische's cysteine-carbazole



**Figure 1** Comparison of the sequence of TcRpiB with those of other Protozoa and prokaryotes

Sequences are shown for the three related trypanosomatids *T. cruzi*, *T. brucei* (gi: 70834348) and *Leishmania major* (gi: 68127548), and two other parasites *Giardia lamblia* (gi: 29248748) and *Entamoeba histolytica* (gi: 56468369) together with the sequence of the crystalized RpiBs from *M. tuberculosis* H37Rv (gi: 57116993) and *E. coli* (gi: 85676843 W3110). Similar residues according to the BLOSUM62 matrix are indicated by different tones of grey, according to the degree of similarity (darker grey indicates more similar residues). Mutated amino acid residues are underlined. *M. tuberculosis* Glu<sup>75</sup> is indicated with an asterisk. Residues that lie within the Rpi active-site pocket are annotated with 1 for the first subunit and with 2 for the second.

method was used [18]. When using Ru5P as a substrate the incubation mixture contained 5  $\mu$ l of the diluted enzyme in buffer A [100 mM Tris/HCl (pH 8.4), 1 mM EDTA and 0.5 mM 2-mercaptoethanol] plus 5  $\mu$ l of Ru5P giving final concentrations between 1 and 10 mM. The final enzyme concentrations in the reaction mixtures typically ranged between 2.5 and 8 ng/ $\mu$ l for wild-type and mutant H102A, and up to 200 ng/ $\mu$ l for the mutant C69A. Incubation was for 15 min at room temperature (25 °C). Following incubation, 15  $\mu$ l of 0.5% cysteinium chloride, 125  $\mu$ l of 75% (v/v) sulfuric acid and 5  $\mu$ l of a 0.1% solution of carbazole in ethanol were added. After 30 min standing at room temperature (25 °C), the  $A_{546}$  was determined. A blank without enzyme was run for each substrate concentration. Reaction linearity was checked in every experiment varying enzyme concentration and time. To estimate the remaining Ru5P a calibration curve was generated; in the assay conditions 0.1  $\mu$ mol of Ru5P gave an  $A_{546}$  of 2.9 in a final reaction volume of 155  $\mu$ l.

Assays with R5P were performed in an incubation mixture containing 17  $\mu$ l of R5P at 10 mM concentration plus 50  $\mu$ l of the enzyme diluted in buffer A; the next steps were as for Ru5P, except for the volumes of cysteinium chloride, sulfuric acid and carbazole, which were increased by 6.7-fold. Thirdly, to determine the enzyme activity on cell-free extracts of the parasite, a coupled enzyme assay following consumption of NADH at 340 nm was used in a mixture containing 50 mM triethanolamine (pH 7.5), 5 mM MgCl<sub>2</sub>, 0.2 mM NADH, 0.025 mM thiamine pyrophosphate, 1 unit of transketolase (EC 2.2.1.1), 1 unit of ribulose 5-phosphate epimerase (EC 5.1.3.1), 20 units of triose-phosphate isomerase (EC 5.3.1.1), 2 units of  $\alpha$ -glycerophosphate dehydrogenase (EC 1.1.1.8) and 1 mM R5P.

### pH profile

The TcRpiB pH profile was determined by incubating the enzyme [5 min at room temperature (25 °C)] with 5 mM MESNA in the following buffers at 100 mM: sodium acetate/acetic acid (pH 4.5–5.5), Mes/NaOH (pH 5.5–6.5), Hepes/HCl (pH 6.3–8.5) and

glycine/NaOH (pH 8.6–11.3). Reactions were started by the addition of R5P at 12.5 mM. The  $A_{290}$  of the commercially available Ru5P was checked between 0.12 and 1.3 mM over the whole pH range tested and did not change significantly.

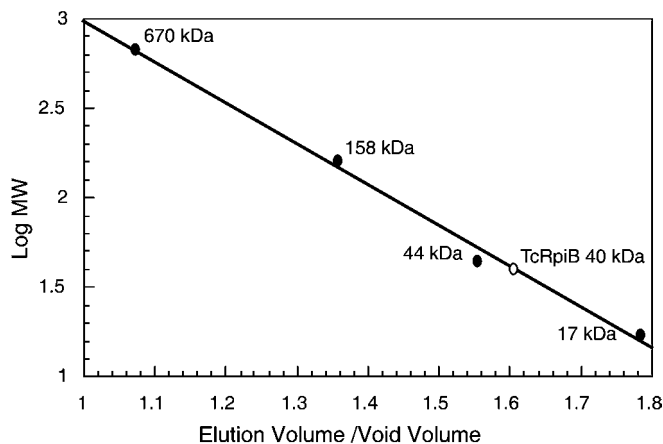
### Site-directed mutagenesis

Site-directed mutagenesis of the *TcRpiB* gene was performed using the QuickChange<sup>TM</sup> site-directed mutagenesis kit and pET28a(+)-TcRpiB as template, according to the instructions provided by the manufacturer. Following mutagenesis, the plasmids were digested with DpnI to destroy the template DNA, *E. coli* BL21 codon Plus (DE3) cells were transformed, and the recombinant proteins produced and purified as described above.

## RESULTS

### Identification of the *TcRpiB* gene

In order to identify Rpi homologues in *T. cruzi*, a BLAST search over the parasite Genome Project database was performed. For this purpose, sequences of RpiAs and RpiBs from diverse sources were used as the query. Although no match against RpiA was found, two open reading frames encoding putative proteins with sequence similarity to RpiB were identified (last searched in February 2006). Both *RpiB* open reading frames were 98% identical with each other and the eleven base replacements found were either silent or conservative except for a histidine to tyrosine residue replacement. Based on this search, specific primers were designed as described in the Materials and methods section. After cloning and sequencing the PCR products, we were able to confirm the existence of one of these open reading frames, which was deposited in GenBank<sup>®</sup> under the accession number DQ782334 and which we have named *TcRpiB*. The coding sequence predicts a 17.4 kDa protein consisting of 159 amino acid residues. Figure 1 shows the alignment of TcRpiB with the sequences deduced from homologous genes obtained from the *T. brucei* and *Leishmania*



**Figure 2** Molecular mass of recombinant TcRpiB as determined by gel-filtration chromatography

Molecular mass markers: bovine thyroglobulin (670 kDa), bovine  $\gamma$ -globulin (158 kDa), chicken ovalbumin (44 kDa) and horse myoglobin (17 kDa).

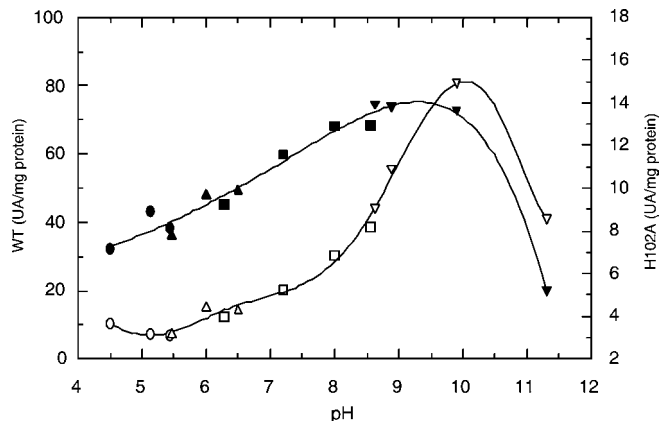
major Genome Projects, as well as those from *G. lamblia*, *E. histolytica*, *E. coli* and *M. tuberculosis*. The Figure also depicts the amino acid residues known to be involved in the active-site formation. RpiBs are homodimeric proteins, the active-site pocket being formed by amino acid residues from both subunits (Figure 1) [6,10,12,19]. Active-site amino acid residues are highly conserved among the family although there is a remarkable change inside the active site of the *M. tuberculosis* enzyme, where the highly conserved Cys<sup>66</sup> (using *E. coli* numbering) is replaced by a glycine residue. Interestingly, structural studies showed that although farther away in the sequence, the side chain of Glu<sup>75</sup> assumes the same position as Cys<sup>66</sup> in the active site of the enzyme [10].

### Expression, purification and properties of recombinant TcRpiB

Recombinant TcRpiB was expressed and purified as described in the Materials and methods section. Gel filtration of TcRpiB showed an active protein peak with an apparent molecular mass of 39.6 kDa, which indicated that the enzyme was a homodimer (Figure 2). In a similar way to other RpiB enzymes, TcRpiB was completely inhibited by 5 mM iodoacetamide, probably due to the reactivity of the highly conserved active site Cys<sup>69</sup> (results not shown). The optimal pH with R5P as the substrate was broad, from 8 to 10 (Figure 3). The  $K_m$  and turnover values for R5P were 4 mM and 12 s<sup>-1</sup> respectively. When using Ru5P as the substrate, the  $K_m$  was 1.4 mM and turnover was 4.7 s<sup>-1</sup>.

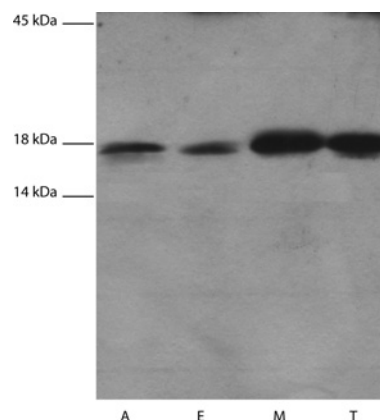
### Rpi activity in *T. cruzi*

Rpi activity was detected in cell-free extracts of amastigotes, epimastigotes, metacyclic trypomastigotes and cell-culture trypomastigotes, being 13 ± 4, 45 ± 5, 43 ± 6 and 40 ± 12 nmol/min per mg of protein respectively. The expression of TcRpiB in the different forms of the parasite was confirmed by Western blotting with an anti-recombinant TcRpiB antibody. All the cell-free extracts showed a single band with an apparent molecular mass of approx. 18 kDa (Figure 4). Although the specific activity was similar in the latter three parasite stages, it did not correlate in the Western blot, where less protein was observed for the epimastigote stage than for the other stages. An explanation for this could be that, since the enzyme assays were performed in cell-free extracts, we cannot rule out the possibility of the presence of



**Figure 3** Specific activity versus pH curves for the wild-type (WT) TcRpiB and H102A mutant

●, WT acetate/acetic; ○, H102A acetate/acetic; ▲, WT Mes/NaOH; △, H102A Mes/NaOH; ■, WT HEPES/HCl; □, H102A HEPES/HCl; ▼, WT glycine/NaOH; ▽, H102A glycine/NaOH.



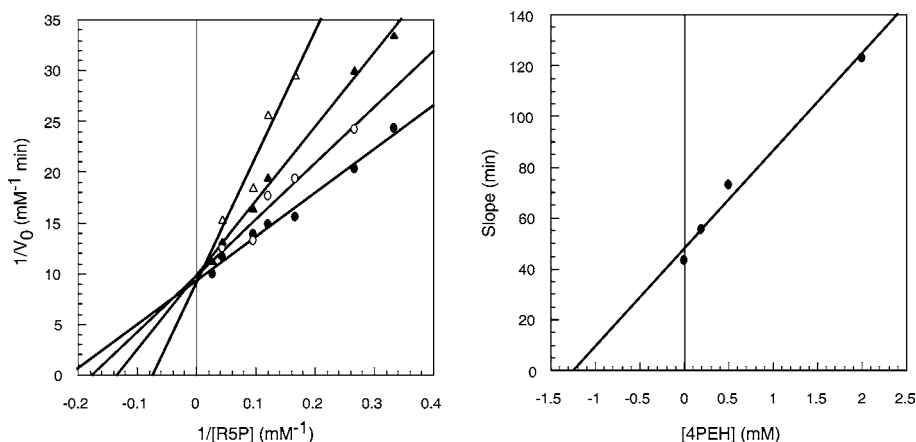
**Figure 4** Differential expression of TcRpiB in different life cycle stages of *T. cruzi*

Western blot of soluble extracts (30  $\mu$ g) of amastigotes (A), epimastigotes (E), metacyclic trypomastigotes (M) and cell-derived trypomastigotes (T). Molecular mass markers: egg ovalbumin (45 kDa), bovine milk  $\beta$ -lactoglobulin (18 kDa) and bovine milk  $\alpha$ -lactalbumin (14 kDa).

phosphatases or other enzymes which might interfere with the assay and which might have different levels in the parasite stages tested (for further analysis of interferences in the Rpi assay see reference [20]).

### TcRpiB mechanism

In an aldose-ketose isomerization reaction, the hydrogen atom which is transferred between C1 and C2 can move by one of two possible mechanisms: a hydride shift or a proton transfer via a 1,2-*cis*-enediol intermediate [21,22]. Enzymes reported to act by the former mechanism require a metal acting as an electrophilic centre so as to promote hydride transfer [23,24]. We might expect that if TcRpiB acts this way, then activity would be affected by metals and metal chelators. Both divalent (Cd<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup>) and monovalent (Na<sup>+</sup> and K<sup>+</sup>) cations, at ranges from 10 nM to 100  $\mu$ M and 50 to 250 mM respectively, were assayed. Furthermore, enzyme samples were treated with EDTA and *o*-phenanthroline at final concentrations of 0.1, 1 and 10 mM. TcRpiB



**Figure 5** Inhibition of TcRpiB

Left-hand panel, double reciprocal plot of initial reaction velocity versus R5P concentration obtained at various concentrations of the inhibitor 4PEH. ●, no inhibitor; ○, 0.2 mM 4PEH; ▲, 0.5 mM 4PEH; and △, 2.0 mM 4PEH. Right-hand panel, secondary plot of slopes against inhibitor concentrations.

activity was not affected in any case, which indicated that TcRpiB was not a metal-dependent enzyme. Because no non-metal dependent aldose-ketose isomerases have been reported to act through a hydride shift mechanism, our results strongly disfavour the possibility of such a mechanism for TcRpiB, giving the way to the *cis*-enediol intermediate mechanism. If TcRpiB acts by an acid-base mechanism involving a *cis*-enediol intermediate, then we would expect that compounds that mimic this intermediate should inhibit the reaction. The possible inhibitory effects of five compounds homologous to the *cis*-enediol intermediary [5PRH (5-phospho-D-ribonohydroxamic acid), 4PEAm (4-phospho-D-erythronamide), 4PEA (4-phospho-D-erythronate), 4PEH (4-phospho-D-erythronohydroxamic acid) and 4PEHz (4-phospho-D-erythronhydrazide)] were tested. The best inhibitor was 4PEH which behaved as a competitive inhibitor, with an  $IC_{50}$  value of 0.7 mM and a  $K_i$  of 1.2 mM (Figure 5). 4PEA, 5PRH and 4PEHz showed a weaker inhibition, and so for these compounds only  $IC_{50}$  values were obtained (5 mM for 4PEA and higher than 10 mM for 5PRH and 4PEHz). The inhibitory effect of most of these compounds contrary to the null effect of metals and metal chelators, strongly suggests a *cis*-enediol intermediary mechanism instead of a hydride transfer.

### Site-directed mutagenesis

Isomerization of R5P to Ru5P requires the open chain form of the sugar instead of the furanose form of R5P, which in fact represents the majority in solution (99%) [25]. Thus it has been proposed that prior to isomerization the enzyme should bind the  $\beta$ -furanose form of R5P and catalyse the opening of the ring. From the analysis of X-ray structures of MtRpiB (*M. tuberculosis* RpiB) in complex with the inhibitors 4PEH and 4PEA, and docking of the substrates, a main role for His<sup>102</sup> and His<sup>138</sup> in the furanose ring opening was suggested. Glu<sup>75</sup> in the *M. tuberculosis* enzyme and Cys<sup>66</sup> from *E. coli* (Cys<sup>69</sup> in *T. cruzi* numbering) have been proposed as the catalytic base for the isomerization [10,11]. Another highly conserved amino acid residue proposed as the catalytic base was His<sup>10</sup> (His<sup>11</sup> in *T. cruzi* numbering) [12].

Based on these considerations, we tested the potential functionality of His<sup>11</sup>, His<sup>102</sup>, His<sup>138</sup> and Cys<sup>69</sup> by site-directed mutagenesis. Kinetic parameters are summarized in Table 1.

The H11A mutant was a protein with a  $K_m$  for R5P 6-fold higher than that for the wild-type enzyme, whereas  $k_{cat}$  was improved

**Table 1** Kinetic parameters of the R5P to Ru5P and Ru5P to R5P isomerizations catalysed by wild-type (WT) and mutant TcRpiBs

ND, not detectable; —, not determined.

	R5P → Ru5P		Ru5P → R5P	
	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )
WT	4 ± 1	12 ± 5	1.4 ± 1.3	4.7 ± 1.5
C69A	ND	ND	ND	ND
H102A	2 ± 1	1	2.5 ± 0.7	10.7 ± 3.4
H138A	10 ± 3	10 ± 3	—	—
H11A	23 ± 1	97 ± 16	—	—

by 8-fold. As the stability of the H11A mutant to freezing and thawing in solution was considerably lower than that of the wild-type enzyme (results not shown) it is possible to suggest that His<sup>11</sup> may play a role in the stabilization of the protein. A role of His<sup>11</sup> in catalysis might be excluded, but the effect on  $K_m$  for R5P may be due either to the protein destabilization, or to a direct effect of the mutation itself on binding. In contrast with the H11A mutant, we were not able to detect activity in the C69A mutant when using either R5P or Ru5P as substrates, which confirms its major role in catalysis. Mutant H138A did not show a difference in  $k_{cat}$  while the  $K_m$  for R5P doubled. As activity was substantially retained in the forward reaction, a main role for His<sup>138</sup> in the ring opening step seems rather unlikely in the case of the TcRpiB enzyme. In contrast with the H138A mutant, the H102A mutant protein showed a 10-fold decrease in  $k_{cat}$  and the  $K_m$  for R5P was little affected, indicating a role of the targeted amino acid residue in catalysis. In order to clarify its responsibility in the ring opening step, kinetic parameters were measured in the reverse reaction, i.e. using Ru5P as the substrate. In this reverse reaction, the  $K_m$  was little affected and  $k_{cat}$  was increased approx. 2-fold over that of the wild-type enzyme. As the His<sup>102</sup> mutant showed an activity severely impaired in the forward reaction but was not affected when using Ru5P as the substrate, a main role in the ring opening step can be assigned to this amino acid residue. When determined with R5P as the substrate, the optimal pH was also changed for the mutant, becoming 10, with a sharp decrease on the acidic side of the curve, in contrast with the curve for the wild-type enzyme (Figure 3). This decrease in the acidic side might be due to the loss

of a catalytically important residue in this sense of the reaction, but a conformational change induced by the mutation cannot be excluded, although the  $K_m$  values for both R5P (at pH 7.6) and Ru5P (at pH 8.4) did not change significantly.

## DISCUSSION

Expression of TcRpiB in the main cellular stages of *T. cruzi* was followed by activity and Western blot assays showing that TcRpiB is constitutively expressed. As in *T. cruzi*, Rpi activity was reported in the different forms of the trypanosomatids *T. brucei* [26], *Leishmania tropica* and *L. major* [27]. Therefore Rpi is not only expressed in the extracellular organisms but also in the intracellular stages of *T. cruzi* and *Leishmania* spp. These trypanosomatids do not have an RpiA homologue in their genome whereas they have an RpiB homologue. In consequence, unless there is another type of Rpi, as yet unknown, the Rpi activity in these organisms corresponds exclusively to RpiB.

TcRpiB is the first protein from the sugar isomerases family LacA/B\_RpiB, which in addition comprises galactose isomerase subunits A and B (Pfam accession number PF02502), characterized from an eukaryotic organism. Solving its reaction mechanism may give insights to be extended to other members of the LacA/B family.

The inhibitory effect of the enediol intermediate homologues, and the lack of effect of metal ions and metal chelators, in addition to previous structural data of the similar MtRpiB complexed with the enediol homologues 4PEH and 4PEA [11] enabled us to assume an isomerization reaction which develops through an enediol intermediary. The inhibition pattern shown by 4PEH, 4PEA, 4PEAm and 4PEHz is identical with that produced by the same compounds on MtRpiB.

To clarify the role of key amino acid residues in the reaction mechanism of TcRpiB, we took advantage from the data available for the more studied EcRpiB (*E. coli* RpiB) and MtRpiB. It has been previously proposed that MtRpiB binds the  $\beta$ -furanose form of R5P. After the sugar is bound, the enzyme catalyses the ring opening step in which His<sup>102</sup> and His<sup>138</sup> would be involved. His<sup>102</sup> has been proposed to donate a proton to ring oxygen atom O4, and the second one would act as a base that accepts a proton from O1H [11]. We think that the present study validates the main role of His<sup>102</sup> in the ring opening step, since the H102A mutant was severely affected in its catalytic properties when using R5P as substrate, but not when using Ru5P.

C69A is a protein devoid of activity in both the forward and reverse reaction proving that Cys<sup>69</sup> is strictly necessary for catalysis. This result is in good agreement with the structural data for MtRpiB and EcRpiB that show Glu<sup>75</sup> in MtRpiB or the corresponding Cys<sup>66</sup> in EcRpiB is well positioned to transfer a proton between C1 and C2 [10–12]. Based on the distance between Asp<sup>9</sup> and His<sup>10</sup> from EcRpiB (Asp<sup>10</sup> and His<sup>11</sup> in TcRpiB numbering) it has been proposed that His<sup>10</sup> could act in concert with Asp<sup>9</sup> as the catalytic base of isomerization [12]. However, subsequent studies with the MtRpiB crystallized with the inhibitors 4PEA and 4PEH show the corresponding histidine residue at a hydrogen bond distance of the phosphate moiety of the inhibitors. Our results clearly ruled out a catalytic role for TcRpiB His<sup>11</sup>, but we cannot discard its participation in substrate binding.

The results in the present paper add to our knowledge of the reaction mechanism of RpiBs. In addition, as there are no homologues of this type of enzyme reported in upper eukaryotic organisms, TcRpiB may be considered a potentially interesting target for the development of new chemotherapeutic drugs.

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