Molecular Characterisation of the First Two Enzymes of the Pentose-Phosphate Pathway of *Trypanosoma brucei*: glucose 6-phosphate dehydrogenase and 6-phosphogluconolactonase

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SUMMARY

Trypanosomatids are parasitic protists which have part of their glycolytic pathway sequestered inside peroxisome-like organelles: the glycosomes. So far, at least one enzyme of the pentose-phosphate pathway has been found to be associated partially with glycosomes. Here we describe how two genes from Trypanosoma brucei, coding for the first two enzymes of the pentose-phosphate pathway, i.e. glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase, were identified by in silico screening of Trypanosome genome-project databases. These genes were cloned and sequenced. Analysis of the lactonase sequence revealed that it contained a C-terminal peroxisome targeting signal in agreement with its subcellular localization in the bloodstream form trypanosome (15% glycosomal and 85% cytosolic). However, the dehydrogenase sequence did not reveal any targeting signal, despite its localization inside glycosomes. The corresponding enzymes have been overexpressed in Escherichia coli, purified and their biochemical characteristics determined.

INTRODUCTION

Glycolysis and the pentose-phosphate pathway (PPP) are the two metabolic pathways in which glucose is catabolized providing the cell with energy (ATP) and reductive power (NADPH), respectively. Although the former process has been well characterized in various species of the pathogenic protozoa of the family Trypanosomatidae, only few details are known about the latter one. Nevertheless, the PPP plays a crucial role in these parasites by generating NADPH which not only serves as a hydrogen donor in biosynthetic processes, but plays possibly also an important role in the defence against the oxidative attack by the infected host. Finally, it
provides also ribose 5-phosphate as nucleic acid precursor and several metabolic intermediates such as fructose 6-phosphate and glyceraldehyde 3-phosphate (for a review, 1).

All enzyme activities involved in the PPP were clearly detected in insect stage (procyclic) forms of *Trypanosoma brucei* and many of them also in the bloodstream form (2) of this parasite which is responsible for African sleeping sickness. So far, one gene coding for 6-phosphogluconate dehydrogenase (EC 1.1.1.44, 6PGDH), the third enzyme of the oxidative branch of the pathway (3) has been cloned and characterized. The enzyme has been overexpressed, purified and its kinetics analyzed, and recently its crystal structure was solved (4, 5, 6). More recently, also the first enzyme of the *T. brucei* PPP, the glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) has been purified and characterized (7). Subcellular fractionation indicated that half of the total cellular activity was present in the cytosol and the other half was found associated with glycosomes, the peroxisome-like organelles of trypanosomes which have as main characterisitic the presence of glycolytic enzymes.

Until recently, no sequence had been reported for the second enzyme of the PPP, 6-phosphogluconolactonase (6PGL, EC 3.1.1.17), which hydrolyses 6-phosphogluconolactone to 6-phosphogluconate. However, based on the homology between products of bacterial *devB* genes, which are often found in the proximity of the G6PDH genes, yeast Sol proteins and the C-terminal part of the hexose-6-phosphate dehydrogenase (H6PDH) which is present in the endoplasmic reticulum of mammalian cells (8), Van Schaftingen and colleagues proposed that *devB* might code for 6PGL (9). Indeed, expression of the human cDNA resulted in a protein which displayed lactonase activity.

Here we report the cloning of the genes encoding the first two enzymes of the oxidative branch of the PPP from *T. brucei*, the expression and preliminary characterisation of the recombinant proteins. The results contribute to a better understanding of the overall trypanosome metabolism and may offer perspectives for the search to new drug targets in the parasite.
MATERIALS AND METHODS

Glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase genes: amplification, sequencing, identification and analysis -- The T. brucei genome project databases at The Sanger Centre (http://www.sanger.ac.uk/Projects/T_brucei/Toolkit/blast_server.shtml) and The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/mdb/tbdb/index.html) were screened with amino-acid sequences of Eschericia coli, yeast and human glucose-6-phosphate dehydrogenase (G6PD_ECOLI, G6PD_YEAST and G6PD_HUMAN (SwissProt database), respectively) using the tblastn program (10). Two short overlapping gel readings (trypA72a4.q1t: 448 bp, trypA67a9.q1t: 346 bp) were found in The Sanger Centre database (March 1999 release) and were 97.8% identical. The same approach was used for 6-phosphogluconolactonase with proteins of bacterial DevB and yeast Sol families (DEVB_ACTAC and SOL1_YEAST) as query sequences. One hit was obtained by screening the TIGR database (June 1999 release; 25D11.TRM13Rev: 668 bp). Sense and antisense oligonuclotides were specified and synthesized (Life Technologies) for each gene: G6PDH - (sense) SG6: 5’-CGATCATTATCTTGGGAAGG-3’ and (antisense) ASG6: 5’-TGAAAGGATCTGGGTAAGG-3’; 6PGL - S6P: 5’-GACTCCACCCTTCCAAC-3’ and AS6P: 5’-GAAAGTCAAAACTTTGCC-3’. These primers were used to amplify by PCR a 211-bp fragment of the g6pdh gene and a 571-bp fragment of the 6pgl gene from T. brucei genomic DNA, with Vent\textsubscript{R} Polymerase (New England Biolabs). PCR products were cloned in the pZErO-2 vector (Invitrogen), sequenced using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech) on a 4000DNA Analysis Sequencer (LI-COR). For both genes, the inserts of a positive recombinant plasmid was purified and used to screen a
λGEM11 *T. brucei* genomic library (11) according to standard methods (12). Positive phages were amplified and their DNAs were purified with the Wizard Lambda Preps DNA purification system (Promega). The genes were subcloned in the pZErO-2 vector and sequenced as described above.

Sequence alignments were performed with the Staden package (13), version 1999. ClustalX (14) was used to obtain alignments and to calculate identity scores. Alignment editing was performed with BoxShade (K. Hofman & M.D. Baron, unpublished: ftp://www.isrec.isb-sib.ch/pub/sib-isrec/boxshade/3.3.1/). Pairwise percentages of identity between the protein sequences were calculated from an uncorrected distance matrix created in ClustalX using the « trees » option after exclusion of all positions with gaps. Protein sequences were retrieved from SwissProt database release 37 (http://www.expasy.ch/sprot/). Secondary structure predictions were made with the program Jpred (15: http://circinus.ebi.ac.uk:8081/).

*Expression and purification of recombinant proteins* -- The complete genes were amplified via PCR using oligonucleotides also containing restriction sites for subcloning in the expression vector. For the *g6pdh* gene, primers were NG6 (sense), containing a *Nde*I site: 5’-CATATGCGAGATTGTAGAAAGGTTG-3’ and CG6 (antisense) with a *Xho*I site: 5’-CTCGAGTTACAAATGATGAAGCTTC-3’; the restriction sites are underlined. The *6pgl* gene was amplified using oligonucleotides N6P with a *Nde*I site: 5’-CATATGTCATTCAAGCCAACCAC-3’ and C6P having a *Bam*HI site: 5’-GGATCCTCAAACCTTTGCCAAAG-3’. Both PCR products were cloned in pZErO-2, sequenced, and subcloned in pET-28b (Novagen) using appropriate enzymes. The *E. coli* BL21(DE3) pLysS strain was then transformed with recombinant pET-28b containing either the *6pgl* or the *g6pdh* gene. The pET28 vectors allowed purification of the recombinant proteins by immobilized metal affinity chromatography (IMAC), based on the synthesis of a His-tagged fusion protein.
For expression and purification of the 6PGL from *T. brucei*, an overnight-grown preculture was used to start a 200-mL culture in LB medium at 37°C with 30 μg·μL⁻¹ kanamycin and 15 μg·μL⁻¹ chloramphenicol. When the OD₆₀₀ reached a value of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, Promega) was added to a final concentration of 1 mM, to induce protein expression, after which growth was continued for 3 hours. For expression of the G6PDH, cells were grown at 30°C in the minimal medium M9 (Life Technologies) supplemented with 0.05% NaCl, 1 mM CaCl, 10 mM MgSO₄, 2% glucose, 30 μg·μL⁻¹ kanamycin and 15 μg·μL⁻¹ chloramphenicol. 1 mM of IPTG was added at an OD₆₀₀ of 0.6, and growth was continued overnight.

Cells were collected by centrifugation (5000 x g, 15 min, 4°C) and resuspended in 10 mL of cell-lysis buffer containing 100 mM triethanolamine-HCl (TEA, pH 7.4), 300 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine-HCl (TCEP, Pierce), 2 mM imidazole and a protease-inhibitor set (Roche). Cells were lysed by two passages through a SLM-Aminco French pressure cell (SLM Instruments Inc.) at 13,000 psi. Nucleic acids were removed by treatment with 250 U Benzonase (Merck) for 30 min at room temperature and protamine sulfate (0.5 mg·mL⁻¹) followed by centrifugation 15 min at 12,000 x g and 4°C. Both G6PDH and 6PGL proteins were further purified from the soluble cell fraction. The supernatent containing 6PGL was directly applied on Talon resin (Clontech) for IMAC purification, whereas G6PDH was first further enriched by precipitation with 50% ammonium sulfate followed by centrifugation (12,000 x g, 15 min, 4°C). The pellet was resuspended in 10 mL of lysis buffer and loaded on the Talon resin. Subsequent purification steps were performed as recommended by the resin manufacturer, with lysis buffer containing 10% glycerol and 10 mM imidazole and at room temperature. Elution of the His₆-tagged proteins was obtained with lysis buffer plus 120 mM imidazole. Purity of G6PDH and 6PGLase was checked by SDS-PAGE followed by Coomassie blue staining. Proteins were stored at 4°C for several weeks in elution buffer but specific activities were
measured after dialysis of the enzymes (3 hours at 4°C, in 100 mM TEA pH 7.4, 1 mM TCEP and the protease inhibitor set).

**Enzymatic assay of the G6PDH and 6PGL from T. brucei** -- Enzymatic activities were measured at 340 nm and 25°C in a 1-mL reaction mixture. G6PDH assays were performed as described (7). Determination of the pH optimum of the enzymes was carried out in acetate-sodium barbital buffers. The $K_m$ of *T. brucei* G6PDH for G6P was determined by varying its concentration between 5 µM and 5 mM, whereas the $K_m$ for NADP$^+$ was measured with concentrations varying between 0.01 and 0.5 mg.mL$^{-1}$.

6PGL activity was measured according to (9), but with several modifications. The concentrations of glucose-6-phosphate (G-6-P) and NADP$^+$ were increased 3 fold (till 0.15 mM G-6-P and 0.6 mM NADP$^+$) to synthesize a sufficient amount of substrate for 6PGL (6-phosphogluconolactone, 6P-G-L) in order to attain a linear relationship between enzyme concentration and reaction rate also at higher protein levels in the assay. Moreover, the reaction buffer was 100 mM TEA pH 7.4 instead of 25 mM Hepes pH 7.1. Linking enzymes were purchased from Roche (yeast G6PDH) and Sigma (yeast 6PGDH), and used at 1.75 U and 0.5 U per assay, respectively. One activity unit (U) is defined as the amount that hydrolyses 1 µmol of substrate (G-6-P or 6P-G-L) per min under these conditions.

**Homogenisation and sub-cellular fractionation** -- Homogenates were obtained by grinding the washed parasite pellets with silicon-carbide abrasive grain (mesh 300) in STE buffer, and a cytoplasmic extract devoid of unbroken cells, nuclei, and debris (post-nuclear fraction) was prepared by centrifugation at 1500 x g for 10 min. For fractionation using isopycnic centrifugation the post-nuclear fractions were layered on top of linear sucrose gradients ranging
from 1.05 to 1.30 g cm$^{-3}$ and centrifuged for 120 min at 200,000 x g in a Beckman VTi 50 rotor. In total 13 fractions of 3 ml each were collected as described by (16).

RESULTS

Identification and cloning of g6pdh and 6pgl genes -- Initial attempts to identify a g6pdh gene in the T. brucei genome by PCR amplification with degenerate oligonucleotides corresponding to conserved regions of eukaryotic and prokaryotic G6PDH sequences were unsuccessful. Thus, we chose to use an in silico gene identification approach through the T. brucei genome sequencing project. Screening of databases using homologous sequences from human, yeast and bacterial G6PDH or 6PGL proteins as query sequences allowed us to identify gel readings obtained from random sheared T. brucei libraries. Two plausible trypanosome G6PDH gene fragments were detected in the The Sanger Centre database. However searching for homologs of 6PGL in this database did not give any significant hit whereas positive matches with a seemingly significant score was obtained by analysing the data available in the TIGR website. To ascertain those identifications, each retrieved sequence was used to screen back general protein databases using blastx. This was especially essential for the 6PGL, since the tblastn identities were only based on very short peptidic fragments (data not shwon). This procedure confirmed the homology also for 6PGL, because the trypanosome fragment coded for a product that recognized in GenBank both the DevB protein homolog of Synechocystis PCC6803 and the Sol1 protein from Saccharomyces cerevisiae (SOL1_YEAST), with 36 and 30% identity, respectively; but now, those identity percentages were related to the predicted amino-acid sequence of the entire fragment (25D11.TRM13Rev - 668 bp).
For the g6pdh gene, although the 2 retrieved shared DNA fragments were larger than 300 bp (trypA72a4.q1t: 448 bp, trypA67a9.q1t: 346 bp), only a smaller fragment (211 bp) was chosen for PCR amplification, in order to prevent mismatches due to possible errors which may be present at the ends of the preliminary sequences retrieved from the database. A similar choice (571 bp) was made for amplification of a fragment of the putative 6pgl gene (25D11.TRM13Rev). The PCR products were subsequently used to screen a genomic library by hybridization and to identify the corresponding recombinant phages. After subcloning and sequencing of phage DNA fragments, open reading frames (ORFs) possibly coding either for G6PDH or 6PGL were revealed.

The putative g6pdh gene is present on T. brucei chromosome I as its sequence was obtained through the current sequencing project of this chromosome by The Sanger Centre. No chromosomal localization could be assigned as yet to the putative 6PGL ORF since it was obtained by an overall genome sequencing approach at TIGR.

For each gene, two independent phages were analysed by restriction with various endonucleases and mapping with the PCR fragment as hybridization probe, subcloning and sequencing. No difference was found between independent phages. The G6PDH ORF contains two potential start codons, 111 nucleotides apart. To get further information about the most plausible start codon, the 5’ end of the mRNA was determined by a reverse transcription-PCR experiment. This was performed by using total RNA purified from procyclic and bloodstream form trypanosomes, as antisense primer an oligonucleotide corresponding to residues 25-30 in Fig. 1 and as sense primer, an oligonucleotide with the sequence of the mini-exon, the spliced-leader sequence present at the 5’ end of all trypanosomatid mRNAs (17, 18). The result (not shown) suggests that the most probable start codon is the second one, giving the shorter protein of 520 amino acids, since the first potential codon, which was only 86 nt downstream of the mini-
exon sequence, would result in an unusually short 5’ untranslated region. The polypeptide has a predicted molecular mass, pI and net charge of 58,990 Da, 7.95 and +1, respectively. The second ATG codon has also been considered as the start codon of the G6PDH when preparing the construct for the expression of the recombinant protein (see below). *T. brucei* 6PGL is a 266-residue protein with a calculated molecular mass of 28,645 Da and a calculated pI of 6.58. Its net charge is -4.

When these two trypanosome PPP proteins were compared with their homologs from other organisms, high identiity scores were obtained (Table I), especially for G6PDH. The *T. brucei* 6PGL is also homologous to glucosamine-6-phosphate deaminase (G6PD, EC 3.5.99.6) from bacteria (21.5% identical with NAGB_ECOLI) and human. The relatedness between these two classes of enzymes was recently noticed (9). Moreover, *T. brucei* lactonase is 24.6% identical to the N-terminal extension of the unusual 107-kDa *Plasmodium falciparum* G6PDH (19).

*Subcellular localisation of 6PGL* -- Both the glucose-6-phosphate dehydrogenase (7) and the lactonase, displayed a dual distribution in *T. brucei* bloodstream forms. In a subcellular fractionation of a cellular extract by sucrose-gradient centrifugation the majority of their activities behaved as a soluble enzyme and remained at the top of the gradient (cf. the cytosolic marker enzyme alanine aminotransferase). However, some ten percent of the total activity of the lactonase and almost 40% of the the glucose-6-phosphate dehydrogenase was associated with particulate material that banded at the same density in sucrose (1.23 g.cm\(^{-1}\)) as did the glycosomal marker enzyme phosphoglycerate kinase (Fig 2).
Structure analysis of G6PDH and 6PGL -- Analysis of the primary structure revealed that the *T. brucei* G6PDH contains the NADP⁺-binding domain $^{75}$GASDLARNK$^{84}$, where underlined residues represent strictly conserved amino acids (20), and the glucose-6-phosphate dehydrogenase signature ($^{209}$DHYLGKE$^{215}$) (see Fig. 1). Also conserved are the residues D209, H210 and H272, which by analysis of the *Leuconostoc mesenteroides* G6PDH were shown to be involved in the catalytic mechanism of the enzyme (21). We used the Jpred program to predict the secondary structure of trypanosome G6PDH (Fig. 1). As control served the *Leuconostoc mesenteroides* and the human G6PDH for which the crystal structures have been determined (1DPG.PDB (21) and 1QKI.PDB (22) respectively). Jpred was able to predict correctly 78% of the secondary structure; errors made were the fusion of 3 α-helices (#10, 11 and 13), the absence of 1 β-sheets (#O) and of 4 α-helices (#14, 15, 16 and 17). α-helix #7 was wrongly assigned by the program, positioning the $^{209}$DH$^{210}$ residues in a short α-helix. Note that human dehydrogenase shares very similar topology to that of *L. mesenteroides* (22).

No motif corresponding to an established peroxisome targeting signals (PTS) of type 1 or 2 was detected in *T. brucei* G6PDH sequence, although previous studies located part of the enzyme’s activity in the glycosome. In contrast, the trypanosome 6PGL contains a PTS-1 variant, the C-terminal tripeptide $^{264}$AKF$^{266}$.

Alignment of the 6PGL amino acid sequences of *T. brucei* and several other organisms (Fig. 3), revealed conservation of the amino acids proposed to be involved in substrate binding (D75, H165 and K223) and those considered as a lactonase signature (R77, F153 and R200) (9). By comparison, the N-terminal extension of the unusual *P. falciparum* G6PDH only contains D and H residues in the proposed substrate-binding motif and the first R residue in the lactonase signature. The function of this N-terminal extension has not been yet elucidated (12).
One insertion was found in the sequence of the \emph{T. brucei} enzyme (\textsuperscript{143}GEAGP\textsuperscript{147}). Interestingly, this sequence contains G and P residues which could allow the formation of a turn in the parasite protein. However, no stretch of positively charged amino-acids was found to be associated with this insertion, a characteristic feature of several glycosomal enzymes (23, 24). In the trypanosome G6PDH no unique insertion was found.

For 6PGL, secondary structure prediction suggested that trypanosomal enzyme could contain 10 \textit{\beta}-sheets and 7 \textit{\alpha}-helices (Fig. 3). Based on the available structure of \emph{E. coli} G6PD (1HOT.PDB) (25), Jpred found 65.2\% of these structural elements. However, four helices were not predicted by the program (# 5, 7, 8 and 11) and two were found as \textit{\beta}-sheet (# 7 and 11). Two \textit{\beta}-sheets were not predicted (#D and C'). Finally, downstream \textit{\beta}-sheet E, Jpred found a short sheet (2 residues, HI) which does not exist in the \emph{E. coli} deaminase. As could be inferred from this comparison (see Fig. 3), \textit{in silico} prediction seems to provide a plausible model of the lactonase structure.

\textit{Overexpression and biochemical studies} -- Both enzymes were expressed in \emph{E. coli} cells as recombinant proteins and purified by IMAC. His-tagged G6PDH could be purified 183 fold to homogeneity (Table II A) but its overexpression required specific growth conditions (see Materials and Methods), whereas 6PGL was expressed under standard conditions, to much higher levels than G6PDH, and required only a 4.1-fold purification (Fig. 4 and Table II B). This suggests that even at high levels, soluble trypanosome 6PGL is not toxic to the bacterial host. After purification, specific activities were 740 and 6753 U.mg^{-1} for G6PDH and 6PGL, respectively (Table III).

The following values were determined for the kinetic parameters for G6PDH: for NADP\textsuperscript{+} the apparent \(K_m = 0.035 \pm 0.004\) mM and for G-6-P the apparent \(K_m = 0.138 \pm 0.030\) mM. G6PDH
was inhibited by 5 mM G-6-P and NADP$^+$ could not be replaced by NAD$^+$ as described previously (7). The pH where activity was optimal, was determined as 8.0.

For His-tagged 6PGL, assay linearity was only and hardly observed when 6PGL concentration ranged from 2.5 to $12.5 \times 10^{-8}$ mg of protein per mL of assay. This prevented us to determine the kinetic properties of the lactonase sufficiently accurate with enzymatically prepared 6-phosphogluconolactone.

**DISCUSSION**

The pentose phosphate pathway is a key metabolic process in the cell because it provides reductive power, ribose 5-phosphate and glycolytic intermediates. The importance of this pathway is illustrated by the fact that, at least in other organisms, the malfunctioning of some of the participating enzymes is detrimental to the cell: several diseases are correlated with mutations in G6PDH (e.g., 26) and accumulation of 6-phosphogluconate, the substrate of 6PGDH, is poisoning the 6-phosphogluconolactonase and consequently blocks glycolysis (27). The importance of this pathway in general explains why we embarked upon a study of the enzymes involved in the oxidative branch of the pentose phosphate pathway in the parasitic protozoan *T. brucei*. This study may lead to the discovery of new potential drug targets.

**G6PDH and 6PGL T. brucei localisation and topologies --** *T. brucei* contains classical G6PDH and 6PGL enzymes, i.e., they do not form a bifunctional enzyme encoded by a single ORF, such as in the case for the human H6PDH protein (8, 9). Our sequence analysis has revealed that the N-terminal extension of the unusual 107-kDa G6PDH from malaria parasite
*Plasmodium falciparum* (19) shares common features with other lactonases. We propose that this unusual dehydrogenase could be a bifunctional enzyme, with the lactonase activity at the N-terminus. However, the differences observed in the substrate-binding motif and in the lactonase signature of the plasmodial enzyme, could reflect a different enzymatic mechanism (substrate affinity or catalytic properties).

Lupianez and colleagues (28) reported two different isoforms of G6PDH in *Trypanosoma cruzi*, characteristic for different stages of the parasite’s life cycle. However, our results suggest the presence of only a single gene of both G6PDH and 6PGL in the *T. brucei* genome. Also biochemical studies did not reveal any evidence for the presence of two isoenzymes in *T. brucei* (7). Yet, G6PDH was found in two different compartments of the cell, cytosol and glycosomes (7) and we demonstrated that 6PGL activity in bloodstream forms of *T. brucei* is also about 85% in the cytosol and 15% in the glycosomes. We showed that the lactonase has the PTS1 motif, whereas no obvious peroxisome-targeting motif could be recognized in G6PDH. Possibly, the latter enzyme has a unique, polypeptide-internal sequence that is responsible for its routing to the glycosome, analogous to previous observations on *T. brucei* triosephosphate isomerase (29) and phosphoglycerate kinase isoenzyme A (30). It has been speculated that such a sequence is not a true topogenic signal, but is rather responsible for the protein’s association with a different protein with an authentic PTS, enabling entry into the organelle by a piggy-back mechanism (31). It has been well established that peroxisomal and glycosomal proteins can enter the organelles in a folded, and multimeric form (32). Whether G6PDH associates with another glycosomal protein, for example 6PGL, remains to be determined.

Both our subcellular fractionation data (7) as well as the presence of a C-terminal PTS-1 signal in the lactonase, are in agreement with a glycosomal location of at least part of the
activities of the two first enzymes of the oxidative branch of the PPP. However, Heise and Opperdoes (7) were unable to find significant amounts inside glycosomes of the third enzyme of the pathway, 6PGDH. Also other enzymes possibly involved in 6-phosphogluconate metabolism such as the Entner-Doudoroff pathway enzymes 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase) could not be detected in either total cellular extracts or glycosomes, nor any sequences homologous to the latter two enzymes were detected in the trypanosome databases (Opperdoes and Van Roy, unpublished). Since there is no evidence that the glycosomal phosphoglucose isomerase becomes inhibited by the accumulation of 6-phosphogluconate inside glycosomes, there must be an alternative, as yet unknown, way for the degradation of this toxic PPP intermediate.

The observation that only a single enzyme can be distributed over different cellular compartments has been reported before, in both trypanosomes and other organisms (for a review, 33). For example, in trypanosomatids, enzymes such as glucose-6-phosphate isomerase, triosephosphate isomerase, and phosphoglycerate kinase may be present in both glycosomes and cytosol, both in a species and in a life-cycle dependent manner (34, 35, 36). The mechanism by which such dual localization in these organisms is achieved and controlled remains so far largely a matter of conjecture (37).

G6PDH and 6PGDH have also been detected into rat liver peroxisomes (38). The reason for the dual subcellular localization of PPP enzymes may be found in the requirement of NADPH not only in the cytosol, but also the glycosomes and peroxisomes. These organelles contain the initial, NADPH-dependent steps of the pathways for the synthesis of ether-lipids and sterols (39, 40, 41). The presence of glycosomal G6PDH is also consistent with the formation of G-6-P from glucose by the glycosomal enzyme hexokinase which is exclusively located in the glycosomes (23). This raises the problem of the intracellular distribution of glycolytic intermediates. It has
been demonstrated that the glycosomal membrane is impermeable to glycolytic intermediates including G-6-P (42). This impermeability seems contradictory with the dual distribution of those enzymes and remains to be elucidated.

In trypanosomes, glycosomal PPP may also play an important role in the protection against activated oxygen species produced by as yet unknown reactions inside the organelles, since neither trypanothione reductase nor hydrogen-peroxide-producing oxidases have been found in them (43).

**Biochemical characteristics** -- Recombinant *T. brucei* G6PDH displays the usual physical characteristics of this dehydrogenase family. Its $K_m$ for G-6-P is 0.138 mM, as previously reported (7). However, the $K_m$ for NADP$^+$ differs clearly (0.035 vs 0.0053 mM) and may be explained by the high purity of the purified protein.

The amino acids involved in the catalytic mechanism of G6PDH, as described for the *L. mesenteroides* dehydrogenase reaction, appear conserved in the *T. brucei* enzyme. H210 may bind the phosphate moiety of G-6-P, D209 may stabilize the positive charge that forms on H272 in the transition state (21).

Preliminary drug-sensitivity assays showed that the trypanocidal drugs suramin and melarsen oxide do not affect *in vitro* activity of the recombinant G6PDH, whereas the antimalarial drug primaquine inhibits with an IC50 of 0.03 mM (D. Cottem & F.R. Opperdoes, unpublished data).

Recombinant *T. brucei* 6-PGL has a very high specific activity compared to the previously described value (2) and we could postulate that this was due to the use of crude cell extract. The $K_m$ for 6-PGL has been reported to 0.83 mM for bovine erythrocyte 6PGL (44), to 7
mM or to 0.080 mM for rat liver (45 and 46 respectively). The validity of such different $K_{m}$ values is questionable, in view of the difficulty of the enzyme assay and the spontaneous hydrolysis of 6-phosphogluconolactone. Thus, we ourselves were unable to perform experiments leading to reproducible values for the $K_{m}$ of trypanosomal lactonase.

It has been assumed that 6-phosphogluconolactones are unstable compounds which hydrolyse spontaneously at physiological pH (47). Therefore, the necessity of a 6PGL to catalyze the reaction in the cells has been questioned. An early study on 6PGL E. coli mutants, suggested that the 6pgl gene should not be essential for the cell and the proposed function of the lactonase was to ensure that the concentration of 6-P-G-L be low enough in the cells rather than to facilitate its further metabolism (47). Recently Rakitzis and Papandreou (48) demonstrated that 6-phosphogluconolactones are highly electrophilic compounds and therefore, may readily react with intracellular nucleophiles exerting untoward metabolic effects. They proposed that the role of the 6PGL in vivo could be the rapid and effective hydrolysis of lactones, preventing ester- and amide-forming reactions involving for example, basic amino acids such as Arg and Lys. Such a role could be particularly critical in the glycosomes of T. brucei where their glycolytic enzymes are clustered (49). Most of these enzymes have a high pI as the result of an excess of Arg and Lys residues clustered on their surface (23, 24). Structure determination of the T. brucei 6PGL is currently being undertaken in order to provide fundamental knowledge about the enzyme’s mode of action.
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Figure 1: Alignment of G6PDHs: 1, *T. brucei* enzyme (AJ249254); 2, yeast protein (G6PD_YEAST); 3, human G6PDH (G6PD_HUMAN) and 4, *L. mesenteroides* dehydrogenase (G6PD_LEUME). Vertical arrowheads highlight conserved residues (D209, H210 and H272) involved in the catalytic mechanism of the enzyme. DHS means G6PDH signature. The thin arrow indicates the antisense primer (residue 25 to residue 30) used for the determination of the most plausible start codon (see text). Horizontal arrows represent predicted β-sheets (letters) while lines are α-helices (numbers). In black, secondary structure elements of the G6PD_LEUME for which the crystal structure is known (1DPG.PDB); in gray, jpred prediction.

Figure 2. Equilibrium density distribution of protein content, sub-cellular marker enzymes and lactonase, obtained from cell-free extracts of *T. brucei* bloodstream form after centrifugation on a linear sucrose gradient. Marker enzymes are hexokinase and phosphoglycerate kinase for the glycosomes, alanine aminotransferase for the cytosol, alpha-mannosidase for the lysosomes and NADP-dependent isocitrate dehydrogenase for the mitochondria. Recoveries (in percent) were: Protein, 94; isocitrate dehydrogenase, 119; hexokinase, 97; alanine aminotransferase, 94; phosphoglycerate kinase, 96 and 6-phosphogluconolactonase, 109.

Figure 3: 6PGL alignment and secondary structure prediction. Black triangles highlight residues proposed to be involved in substrate binding. Residues thought to be the lactonase signature are marked with a dot. 1, *T. brucei* 6PGL (AJ249255); 2, *Syneccystis* sp. DevB protein; 3, yeast Sol4 protein (Y_YEAST; 4, human 6PGL (AJ243972); 5, C-terminal extension of the human H6PDH (NM_004285); 6, *E. coli* glucosamine 6-phosphate deaminase (G6PD, NAGB_ECOLI) and 7, N-terminal part of malarial G6PDH (X74988). Secondary structure elements are shown as in Fig. 1; numbering is based on the published structure (1HOT.PDB, Oliva et al., 1995).
Figure 4: Electrophoretic analysis of the purification steps of *T. brucei* G6PDH and 6PGL from BL21(DE3)pLysS strain. Mr: molecular mass markers (in kDa); lanes 1 and 4: lysates of non-induced controls cells; lanes 2 and 3: soluble cell fraction after French pressure cell lysis, treatment with nuclease and protamine sulfate followed by centrifugation; lanes 3 and 6: fractions after IMAC following elution with 120 mM imidazole.
Table I: Percentage of identity between whole *T. brucei* proteins and homologous sequences

| G6PDH          | T. brucei G6PDH | T. brucei 6PGL |
|----------------|-----------------|---------------|
| **Monofunctional:** |
| *L. mesenteroides*, G6PD_LEUME | 36.1           |               |
| *E. coli*, G6PD_ECOLI           | 41.9           |               |
| *S. cerevisiae*, G6PD_YEAST     | 55.1           |               |
| G6PD_HUMAN                  | 53.4           |               |
| **Bifunctional:**            |                 |               |
| Human hexose-6-phosphate dehydrogenase, NM_004285 | 33.3 (N-terminus) | 30.0° (C-terminus) |
| *P. falciparum*, X74988        | 40.3 (C-terminus) | 24.6° (N-terminus) |

| 6PGL   |                 |               |
|--------|-----------------|---------------|
| *Synechocystis* sp., DEVB_SYNY3 | 37.7           |               |
| *A. actinomycetemcomitans*, DEVB_ACTAC | 29.2           |               |
| Yeast, SOL4_YEAST                 | 29.2           |               |
| Human, AJ243972                    | 36.9           |               |
| *E. coli* glucosamine-6-phosphate deaminase, NAGB_ECOLI | 21.5           |               |

a: C-terminal extension of the human H6PDH started from position 551 to 790 and N-terminal extension of the unusual malarial G6PDH, from position 1 to 240.
Table II: Purification of recombinant *T. brucei* G6PDH (A) and 6PGL (B) from *E. coli* BL21(DE3) pLysS

### A

| Step                                      | Protein (mg) | Total activity (U) | Specific activity (U.mg\(^{-1}\)) | Yield (%) | Purification factor (-fold) |
|-------------------------------------------|--------------|--------------------|-----------------------------------|-----------|-----------------------------|
| Supernatant after treatment with nuclease and protamine sulfate | 2.3          | 144                | 48                                | 100       |                             |
| Eluate                                    | 0.06         | 148                | 740                               | 30        | 183                         |

### B

| Step                                      | Protein (mg) | Total activity (U) | Specific activity (U.mg\(^{-1}\)) | Yield (%) | Purification factor (-fold) |
|-------------------------------------------|--------------|--------------------|-----------------------------------|-----------|-----------------------------|
| Supernatant after treatment with nuclease and protamine sulfate | 3.1          | 5093               | 1643                              | 100       |                             |
| Eluate                                    | 0.55         | 3714               | 6753                              | 73        | 4.1                         |
Table III: comparison of previously published data on G6PDH and 6PGL:

|        | Cronin et al., 1989 | Heise & Opperdoes, 1999 | this study |
|--------|---------------------|-------------------------|------------|
| G6PDH  |                     |                         |            |
| Specific activity (U.mg$^{-1}$) | ~ 0.017 | 14 | 740 |
| $K_m$ G-6-P (mM) | ND | 0.138 | 0.138 |
| $K_m$ NADP (mM) | ND | 0.0053 | 0.035 |
| 6PGL   |                     |                         |            |
| Specific activity (U.mg$^{-1}$) | 0.040 | 6753 | 710 |

ND: not determined
Fig2

- Alanine amino-transferase (93.8)
- Glucose-6-phosphate dehydrogenase (100.0)
- Phosphoglycerate kinase (96.0)
- 6-phosphogluconolactonase (108.9)
Fig 4.

|      | G6PDH | 6PGL |
|------|-------|------|
| Mr   | 1     | 2    | 3    | 4    | 5    | 6    | Mr   |
| 94   |       |      |      |      |      |      |      |
| 67   |       |      |      |      |      |      |      |
| 43   |       |      |      |      |      |      |      |
| 30   |       |      |      |      |      |      |      |
Molecular characterisation of the first two enzymes of the pentose-phosphate pathway of trypanosoma brucei

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