Roles of triosephosphate isomerase and aerobic metabolism in *Trypanosoma brucei*

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Kinetoplastid protozoa compartmentalize the first seven enzymes of glycolysis and two enzymes of glycerol metabolism in a microbody, the glycosome. While in its mammalian host, *Trypanosoma brucei* depends entirely on glucose for ATP generation. Under aerobic conditions, most of the glucose is metabolized to pyruvate. Aerobic metabolism depends on the activities of glycosomal triosephosphate isomerase and a mitochondrial glycerophosphate oxidase, and on glycerophosphate ↔ dihydroxyacetone phosphate exchange across the glycosomal membrane. Using a combination of genetics and computer modelling, we show that triosephosphate isomerase is probably essential for bloodstream trypanosome survival, but not for the insect-dwelling procyclics, which preferentially use amino acids

as an energy source. When the enzyme level decreased to about 15% of that of the wild-type, the growth rate was halved. Below this level, a lethal rise in dihydroxyacetone phosphate was predicted. Expression of cytosolic triosephosphate isomerase inhibited cell growth. Attempts to knockout the trypanosome alternative oxidase genes (which are needed for glycerophosphate oxidase activity) were unsuccessful, but when we lowered the level of the corresponding mRNA by expressing a homologous double-stranded RNA, oxygen consumption was reduced four-fold and the rate of trypanosome growth was halved.

Key words: glycolysis, glycosome, metabolic control, RNA interference, trypanosome alternative oxidase.

INTRODUCTION

Salivarian trypanosomes are extracellular parasites of the blood and tissue fluids of mammals. In Africa, the parasites are transmitted by tsetse flies, which are restricted to the sub-Saharan region. Some subspecies of *Trypanosoma brucei* cause sleeping sickness in man; approx. 200 000 new infections occur every year (see <http://www.who.int/health-topics/aftrtryps.htm>). The infection is lethal unless treated, and the few existing drugs have severe side-effects. The parasites also have a significant economic impact through infection of livestock.

The free-energy metabolism of *Trypanosoma brucei* in the mammal is extremely simple, consisting only of glycolysis. Under aerobic conditions, most glucose is metabolized to pyruvate (PYR), although about 10% is converted into glycerol (Scheme 1); both products are excreted. Since the fluxes through side pathways are negligible, it has been possible to generate a computer model of the entire pathway. This model is able to predict the consequences of adjusting external conditions or the levels of particular enzymes [1]. The model predicts that, in the presence of 5 mM glucose, the normal serum level, most

control of the glycolytic flux is exerted by the plasma-membrane glucose transporter, but some other enzymes also contribute to control [2].

In bloodstream trypanosomes, the first seven enzymes of glycolysis and two enzymes of glycerol metabolism are confined within a peroxisome-like organelle, the glycosome (reviewed in [3–5]) (Scheme 1). The reactions interconverting ATP and ADP are predicted to balance within the glycosome, and genetic manipulations leading to a partial diversion of ATP generation to the cytosolic compartment cause severe growth defects, suggesting that the glycosomal membrane may be impermeable to ATP and ADP [6]. In contrast with the situation in other cells, neither hexokinase nor phosphofructokinase is susceptible to regulation by any compounds that are known to regulate their counterparts in other cell types. Computer reconstructions indicate that the glycosomal membrane is essential because it prevents these enzymes from obtaining access to the high cytosolic ATP concentration [7], which could lead to runaway hexose phosphorylation and depletion of cellular phosphate pools.

The membrane of yeast peroxisomes is impermeable to NAD⁺/NADH [8], and all observations so far are consistent

Abbreviations used: Abbreviations used in the text, and/or in equations and Scheme 1: general; SHAM, salicylhydroxamic acid; ds, double-stranded; gene names: *NEO*, neomycin phosphotransferase marker (fused to a short leader from trypanosome tubulin); *HYG*, hygromycin-resistance marker; *BLE*, bleomycin-resistance marker; *BST*, blasticidin-resistance marker; *TPI*, triosephosphate isomerase gene; *TPI^{TI}*, tetracycline-inducible triosephosphate isomerase gene; *TAO*, trypanosome alternative oxidase gene; *TETR*, tetracycline repressor gene; metabolic intermediates: Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; Fru-1,6-P₂, fructose 1,6-bisphosphate; GA-3-P, glyceraldehyde 3-phosphate; PYR, pyruvate; DHAP, dihydroxyacetone phosphate; BPGA, bisphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Gly-3-P, glycerol 3-phosphate; enzymes: ALD, aldolase; HXK, hexokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase [GAPDH1 is glycosomal and GAPDH2 is cytosolic]; GDH, glycerol-3-phosphate dehydrogenase; GLYK, glycerol kinase; GPO, glycerol-3-phosphate oxidase; PGI ('phosphoglucose isomerase'), glucose-6-phosphate isomerase; PGK, phosphoglycerate kinase; PYK, pyruvate kinase; PFK, phosphofructokinase; TAO, trypanosome alternative oxidase; TPI, triosephosphate isomerase; ZMBH, Zentrum für Molekularbiologie der Universität Heidelberg.

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Scheme 1 Glycolysis in bloodstream trypanosomes

Normally, the entire pathway with the exception of GPO and the reactions after phosphoglycerate kinase (PGK) is inside the glycosome, and the products are 90% PYR and 10% glycerol. The pathways shown with broken lines indicate the likely effects of the expression of *S. cerevisiae* TPI (*ScTPI*). The cytosolic GAPDH2 isoenzyme is constitutively expressed. For abbreviations, see the abbreviations footnote at the beginning of this paper.

with the notion that the glycosomal membrane is similarly impermeable [5]. Within the glycosome, glyceraldehyde 3-phosphate (GA-3-P) is oxidized to 1,3-bisphosphoglycerate (1,3-BPGA) with concomitant reduction of NAD^+ to NADH (Scheme 1). The balance of reducing equivalents within the glycosome is maintained by glycerol phosphate dehydrogenase, which reduces dihydroxyacetone phosphate (DHAP) to glycerol phosphate with concomitant re-oxidation of NADH. The glycerol phosphate produced can either be converted to glycerol (generating ATP within the glycosome) or exported to the cytosol. Under aerobic conditions, a mitochondrial glycerol-3-phosphate oxidase [GPO, consisting of a flavoprotein glycerol-3-phosphate dehydrogenase (GDH) and an alternative oxidase, trypanosome alternative oxidase (TAO)] reconverts the glycerol 3-phosphate into DHAP, which returns to the glycosome for conversion into GA-3-P by triosephosphate isomerase (TPI). The export of glycerol 3-phosphate therefore results in the export of reducing equivalents from the glycosome. If one glucose molecule were converted entirely into PYR, the yield from glycolysis would be two ATP molecules, whereas if oxidation of glycerol 3-phosphate were completely inhibited, the yield would be halved (reviewed in [5,9]).

The mitochondrial GPO can be inhibited by salicylhydroxamic acid (SHAM). Between 1976 and 1980 it was demonstrated that SHAM alone does not kill bloodstream trypanosomes. The SHAM-treated parasites could, however, be killed by including glycerol, which inhibits the dephosphorylation of glycerol 3-phosphate by mass action [10,11]. These results were confirmed more recently in experiments involving another inhibitor of the oxidase, ascofuranone [12]. The investigations described in the present paper started with attempts to delete the genes encod-

ing TPI (*TPI*). We anticipated that the resulting trypanosomes should be viable, but have reduced energy yield from glycolysis.

MATERIALS AND METHODS

Parasites and culture

Bloodstream trypanosomes of strain TREU 427 were continuously cultured at 37 °C in HMI-9 with 10% (v/v) newborn-calf serum. Often phleomycin-resistant parasites expressing the *tet* repressor (integrated pHD449, genotype *BLE TETR*, where *TETR* is the tetracycline repressor gene) were used [13]. These parasites are metabolically indistinguishable from the wild-type. Procyclic trypanosomes (427 strain) were cultivated in SDM-79 [13]. Metabolic experiments were performed using a modified minimal essential medium [6]. To obtain anaerobic conditions, the medium was supplemented with 40 mM ascorbate and 100 units/ml ascorbate oxidase [14], and the vessels were filled with argon.

Transfection methodology was as previously described [13,15]; concentrations of selecting drugs were as follows: for bloodstream cells, 0.2 µg/ml phleomycin, 15 µg/ml hygromycin, 2 µg/ml G418, 4–6 µg/ml blasticidin and 100–500 ng/ml tetracycline; for procyclic cells, 50 µg/ml hygromycin and 25 µg/ml G418. To determine the sensitivity to SHAM (Sigma) a 1 M solution in dimethylformamide was diluted to 1 mM or less in culture medium. Diluted solvent alone had no effect. Drug was added to duplicate or triplicate 0.5–1 ml cultures.

Plasmid constructs

The *T. brucei* *TPI* coding region was excised from the cDNA clone pG/TIMTb and cloned into the tetracycline-inducible expression vector pHD615 [13] as a *HindIII*–*BamHI* fragment (pHD832). The resulting gene encodes a TPI with 22 additional amino acids at the C-terminus of TPI; this did not compromise enzyme activity or compartmentation. Oligonucleotides used for fragment amplification are listed in Table 1. Reconstructed sequences, DNA and details of all plasmids used in the present study are available from C. C. and described in [16]. The *Saccharomyces cerevisiae* *TPI* gene was amplified from genomic DNA [kindly given by Ralf Jansen, Zentrum für Molekularbiologie der Universität Heidelberg (ZMBH), Heidelberg, Germany] and cloned into pHD615. The 3'- and 5'-untranslated regions of *TPI* were amplified from a plasmid clone, and placed either side of hygromycin (*HYG*) (pHD795), G418 (*NEO*) (pHD794) or blasticidin (*BST*) (pHD888) resistance cassettes. Regions 3' and 5' to, and within, the trypanosome alternative oxidase gene, *TAO* [17], were amplified using trypanosome genomic DNA as template. For RNA interference, the first 816 nt of the *TAO* gene were amplified with the oligonucleotides CZ1428 and CZ1429. The ATG initiator codon was mutated to ATT in order to avoid the translation of a truncated form of the TAO. The PCR product was digested with *XhoI* and *HindIII* and cloned in the pZJM vector [18], yielding the vector pHD1126. Transfection of bloodstream 328.114 [19] parasites using *NotI*-linearized pHD1126 yielded no transformants. We then used the RNA interference strategy developed by Shi et al. [20] in which sense and antisense fragments are placed at opposite ends of a 'stuffer' fragment. Plasmid pHD1126 was digested either with *BglII* and *HindIII* or *EcoRI* and *BglII*. The purified fragments were ligated to the stuffer fragment previously digested with *HindIII* and *EcoRI* and the ligation reaction was separated in an agarose gel. The fragment corresponding to the stuffer flanked by the sense and antisense versions of the original PCR product was purified and ligated into *BamHI*-digested pHD1145 (a

Table 1 Oligonucleotides used in the present study

Abbreviations used: UTR, untranslated region.

Oligonucleotide	Sequence
CZ674	GCGGAGCTCGGAGATGAGAAGCCAGAAGC (<i>TPI</i> 5'-UTR sense)
CZ675	GCGTCTAGAGGCTTCTGTTGCTTGC (<i>TPI</i> 5'-UTR antisense)
CZ676	GCGGGATCCGCTGGACATCATCAAGCC (<i>TPI</i> 3'-UTR sense)
CZ677	GCGCTCGAGGGGGGAATATAATGAAGGC (<i>TPI</i> 3'-UTR antisense)
CZ1159	TTCGGGCCGCCCTTGGCATACGAAAAG (<i>TAO</i> 5'-UTR sense)
CZ1160	TGCTCTAGATCCTCAAGGATGATGTTT (<i>TAO</i> 5'-UTR antisense)
CZ1161	CGCGGATCCGCTGGATGCCATCAG (<i>TAO</i> 3'-UTR sense)
CZ1162	CCGCTCGAGGCGTTACACATTTTCACT (<i>TAO</i> 3'-UTR antisense)
CZ1221	AAATATGCGGCCGCCGAAAAGAAATACAACATTAAGTG (<i>TAO</i> 5'-UTR sense)
CZ1222	CTAGTCTAGAGGTACGAAACAATCCTCAAGG (<i>TAO</i> 5'-UTR antisense)
CZ1223	AAATATGCGGCCGCCGAGGATTGTTTCGTAACACGCG (<i>TAO</i> 5'-UTR sense)
CZ1224	CTAGTCTAGACTGAGACGGTTCAGTTGAGTGTGTCCTCC (<i>TAO</i> 5'-UTR antisense)
CZ1226	GCTAAGCTTAAGATGGCTAGAACTTTCTTT (<i>ScTPI</i> 5' sense)
CZ1227	GCAGGATCCTTAGTTTCTAGAGTTGATG (<i>ScTPI</i> 3' antisense)
CZ1428	GACGCTCGAGGAT77GTTTCGTAACACGCGATCG (<i>TAO</i> dsRNA)
CZ1429	GACCAAGCTTAACGACACGGTGCTCCGCTTCG (<i>TAO</i> dsRNA)

pHD677 [13] derivative from which the backbone-derived T7 promoter was removed). The resulting plasmid, pHD1155, was linearized with *NotI* and used to transfect bloodstream 449 cells. Transformants were selected in 5 µg/ml of hygromycin and cloned by limiting dilution. RNA interference induction was achieved by adding tetracycline to the medium at a concentration of 100 ng/ml.

Protein, RNA and metabolite analysis

Enzyme assays were done as described in [21]. Western blots, cell fractionation and metabolite analysis were as described in [6]. For metabolite analysis, each sample contained at least 1×10^9 trypanosomes. Western-blot quantification was effected by scanning the blots followed by analysis using NIHImage software. For Northern-blot analysis, 25 µg of total RNA was electrophoresed in a 1% agarose/formaldehyde/TAE (Tris/acetate/EDTA buffer) gel, transferred to a Nytran neutral membrane (Schleicher and Schuell) and probed with a radiolabelled probe (the PCR product used to make the RNA interference vector) following the manufacturer's instructions. Oxygen consumption by trypanosomes was polarographically determined at 37 °C using a closed Clark-type oxygen electrode. For each experiment, 6.5×10^7 living cells were added to HMI-9 medium. SHAM was dissolved in DMSO, but added to the incubation medium such that the final DMSO concentration was never higher than 1%. The final concentration of SHAM was 100 µM. The O₂ concentration in the incubation medium at 37 °C was taken to be 225 µM.

Extension of the metabolic model to include TPI kinetics

Time-dependent behaviour of glycolysis

To predict the effects of inhibition of TPI, the model of *T. brucei* glycolysis that was described previously [1,2] was extended. The measured kinetics of glucose-6-phosphate isomerase (PGI) and TPI were incorporated in the model instead of assuming that both were at equilibrium. Since the stoichiometry was not changed compared with the previous version [2], the moiety conserved sums did not change either.

The following set of differential equations (for abbreviations, see the footnote) describe the time-dependent behaviour of

glycolysis. Metabolite concentrations are in mM and the time is given in minutes. The enzyme rates, v , are expressed as nmol·min⁻¹·mg of protein⁻¹, and the glycosomal volume, V_g , and total cell volume, V_{tot} , are expressed as in µl·mg of protein⁻¹.

$$\frac{d[\text{Glc}]_{in}}{dt} = \frac{v_{\text{glucose transport}} - v_{\text{HXK}}}{V_{tot}} \quad (1)$$

$$\frac{d[\text{Glc-6-P}]_g}{dt} = \frac{v_{\text{HXK}} - v_{\text{PGI}}}{V_g} \quad (2)$$

$$\frac{d[\text{Fru-6-P}]_g}{dt} = \frac{v_{\text{PGI}} - v_{\text{PFK}}}{V_g} \quad (3)$$

$$\frac{d[\text{Fru-1,6-P}_2]_g}{dt} = \frac{v_{\text{PFK}} - v_{\text{ALD}}}{V_g} \quad (4)$$

$$\frac{d[\text{GA-3-P}]_g}{dt} = \frac{v_{\text{ALD}} + v_{\text{TPI}} - v_{\text{GAPDH}}}{V_g} \quad (5)$$

$$\frac{d[\text{DHAP}]_g}{dt} = \frac{v_{\text{ALD}} - v_{\text{TPI}} - v_{\text{GDH}} + v_{\text{GPO}}}{V_{tot}} \quad (6)$$

$$\frac{d[1,3\text{-BPGA}]_g}{dt} = \frac{v_{\text{GAPDH}} - v_{\text{PGK}}}{V_g} \quad (7)$$

$$\frac{d[\text{N}]}{dt} = \frac{v_{\text{PGK}} - v_{\text{PYK}}}{V_{tot}} \quad (8)$$

$$\frac{d[\text{PYR}]_c}{dt} = \frac{v_{\text{PYK}} - v_{\text{pyruvate transport}}}{V_c} \quad (9)$$

$$\frac{d[\text{NADH}]_g}{dt} = \frac{v_{\text{GAPDH}} - v_{\text{GDH}}}{V_g} \quad (10)$$

$$\frac{d[P]_g}{dt} = \frac{-v_{\text{HK}} - v_{\text{PFK}} + v_{\text{PGK}} - v_{\text{GLYK}}}{V_g} \quad (11)$$

$$\frac{d[P]_c}{dt} = \frac{v_{\text{PYK}} - v_{\text{ATP utilization}}}{V_c} \quad (12)$$

In these equations the subscripts _g and _c denote the glycosomal and the cytosolic concentration respectively. [Glc]_{in} is the intracellular glucose concentration, which is assumed to be equal in

the cytosol and the glycosome. [DHAP] represents the average DHAP concentration:

$$[\text{DHAP}] \equiv ([\text{DHAP}]_g \cdot V_g + [\text{DHAP}]_c \cdot V_c) / V_{\text{tot}} \quad (13)$$

[N] is the pool of glycosomal and cytosolic 3-phosphoglycerate (3-PGA) and cytosolic 2-phosphoglycerate (2-PGA) and phosphoenolpyruvate (PEP):

$$[\text{N}] \equiv ([3\text{-PGA}]_g \cdot V_g + [3\text{-PGA}]_c \cdot V_c + [2\text{-PGA}]_c \cdot V_c + [\text{PEP}]_c \cdot V_c) / V_{\text{tot}} \quad (14)$$

P_g and P_c denote the sums of high-energy phosphates in the glycosome and the cytosol respectively:

$$P_g \equiv 2[\text{ATP}]_g + [\text{ADP}]_g \quad (15)$$

$$P_c \equiv 2[\text{ATP}]_c + [\text{ADP}]_c \quad (16)$$

The steady-state metabolite concentrations and fluxes were calculated by setting all time derivatives to zero and solving the above set of non-linear equations with a Marquardt–Levenberg algorithm [21a]. The simulations were performed with the program MLAB (Civilized Software, Bethesda, MD, U.S.A.).

Equilibrium of DHAP $\leftrightarrow$ glycerol 3-phosphate (Gly-3-*P*) exchange

As previously the exchange of DHAP and Gly-3-*P* was assumed to work at equilibrium [2]. Since TPI was not assumed to work at equilibrium any more, the calculation of the glycosomal and cytosolic concentrations of Gly-3-*P* and DHAP from the independent variables was simplified. These four dependent variables were calculated by solving a set of four equations. These were eqn (13), the moiety conservation relations for glycosomal and cytosolic bound phosphates respectively, and the equilibrium equation of the exchange process:

$$\begin{aligned} &[\text{Gly-3-}P]_g + [\text{DHAP}]_g + [\text{Glc-6-}P]_g + [\text{Fru-6-}P]_g \\ &\quad + 2[\text{Fru-1,6-}P_2]_g + [\text{GA-3-}P]_g + [1,3\text{-BPGA}]_g \\ &\quad + 2[\text{ATP}]_g + [\text{ADP}]_g = C_{4,\text{antiporter}} \end{aligned} \quad (17)$$

$$[\text{Gly-3-}P]_c + [\text{DHAP}]_c = C_{5,\text{antiporter}} \quad (18)$$

$$\frac{[\text{Gly-3-}P]_c [\text{DHAP}]_g}{[\text{Gly-3-}P]_g [\text{DHAP}]_c} = K_{\text{eq,antiporter}} = 1 \quad (19)$$

This led to the following solution:

$$[\text{DHAP}]_c = \frac{C_{5,\text{antiporter}} \cdot [\text{DHAP}] \cdot (V_c/V_g + 1)}{C_{5,\text{antiporter}} \cdot V_c/V_g + R} \quad (20)$$

in which:

$$\begin{aligned} R &\equiv [\text{Gly-3-}P]_g + [\text{DHAP}]_g \\ &= C_{4,\text{antiporter}} - [\text{Glc-6-}P]_g - [\text{Fru-6-}P]_g - 2[\text{Fru-1,6-}P_2]_g \\ &\quad - [\text{GA-3-}P]_g - [1,3\text{-BPGA}]_g - 2[\text{ATP}]_g - [\text{ADP}]_g \end{aligned} \quad (21)$$

where Glc-6-*P* is glucose 6-phosphate, Fru-6-*P* is fructose 6-phosphate and Fru-1,6-*P*₂ is fructose 1,6-bisphosphate. Subsequently, [Gly-3-*P*]_c was calculated from eqn (18), [DHAP]_g from eqn (13) and [Gly-3-*P*]_g from eqn (17).

The kinetic equations and parameters have been listed previously [1]. The modifications described in [2] were used. For PGI a reversible Michaelis–Menten equation [22] was implemented:

$$v_{\text{PGI}} = \frac{V_{\text{max}}^r \cdot \frac{[\text{Glc-6-}P]_g}{K_{\text{m,Glc-6-}P}} - V_{\text{max}}^f \cdot \frac{[\text{Fru-6-}P]_g}{K_{\text{m,Fru-6-}P}}}{1 + \frac{[\text{Glc-6-}P]_g}{K_{\text{m,Glc-6-}P}} + \frac{[\text{Fru-6-}P]_g}{K_{\text{m,Fru-6-}P}}} \quad (22)$$

in which $K_{\text{m,Glc-6-}P}$ was 0.4 mM (F. R. Oppendoes, personal communication) and $K_{\text{m,Fru-6-}P}$ was 0.12 mM [23]. The measured maximum specific activity of PGI in the direction of Fru-6-*P* conversion was 565 units/mg of enzyme (F. R. Oppendoes, personal communication). Since PGI represents 0.15% of the total cell protein [24], V_{max}^r (r means reverse) is 848 nmol·min⁻¹·mg of cell protein⁻¹. The equilibrium constant ($[\text{Fru-6-}P]/[\text{Glc-6-}P]_{\text{eq}}$) of the enzyme is 0.29. The Haldane relation then reveals that V_{max}^f (f means forward) should equal V_{max}^r .

The kinetics of TPI were also described by a reversible Michaelis–Menten equation:

$$v_{\text{TPI}} = \frac{V_{\text{max}}^f \cdot \frac{[\text{DHAP}]_g}{K_{\text{m,DHAP}}} - V_{\text{max}}^r \cdot \frac{[\text{GA-3-}P]_g}{K_{\text{m,GA-3-}P}}}{1 + \frac{[\text{DHAP}]_g}{K_{\text{m,DHAP}}} + \frac{[\text{GA-3-}P]_g}{K_{\text{m,GA-3-}P}}} \quad (23)$$

in which $K_{\text{m,GA-3-}P}$ is 0.25 mM and $K_{\text{m,DHAP}}$ is 1.2 mM [25]. The maximum catalytic-centre activity ('turnover number') of a TPI subunit with DHAP as the substrate is 6.5×10^4 min⁻¹, the subunit molecular mass is 27 kDa [25] and TPI represents 0.035% of the total cell protein [24]. Together, these data yield a V_{max}^f of 842 nmol·min⁻¹·mg of cell protein⁻¹. Finally, V_{max}^r was reported to be 5.7 times V_{max}^f [24].

The V_{max} of PYR export from the cells was 200 nmol·min⁻¹·mg of cell protein⁻¹, as in [2], where this modification should have been mentioned as well.

In the simulations described in the present paper, glycerol kinase is always included in the differential equations.

RESULTS

The TPI gene is essential

This study was initiated by an attempt to delete the *TPI* gene from *T. brucei* by homologous recombination. Deletion of one allele of *TPI*, and replacement of the gene with either the G418-resistance or hygromycin-resistance cassettes, to yield the genotypes $\Delta tpi::NEO/TPI$ or $\Delta tpi::HYG/TPI$ (confirmed by blot hybridization) was unproblematic [16]. The $\Delta tpi::NEO/TPI$ trypanosomes were now re-transfected with the knockout construct bearing the *HYG* gene. If both selecting drugs were included in the medium, no clones were obtained. As a positive control, G418 was omitted from the medium, but hygromycin was included. Under these conditions, transformants were obtained at high frequency, but in all cases the *HYG* gene had replaced the *NEO* gene, leaving the remaining *TPI* gene intact [16]. Analogous results were obtained upon attempts to transfect the $\Delta tpi::HYG/TPI$ trypanosomes with the *NEO* marker [16]. We concluded that either TPI is essential in bloodstream trypanosomes, or its depletion is so detrimental as to prevent isolation of knockout clones.

As an additional positive control, similar experiments were conducted in procyclic trypanosomes. In this life-cycle stage there is active mitochondrial oxidative phosphorylation, enabling the use of amino acids as carbon and energy source, and reliance on glycolysis is reduced. The parasites were grown with proline as the main free energy source, by-passing glycolysis. The $\Delta tpi::HYG/\Delta tpi::NEO$ knockout lines were obtained without difficulty and with no apparent growth defects [16].

TPI depletion

Failure to delete a gene could be caused by very slow growth of the resulting mutants. To characterize cells with reduced TPI, we

Figure 1 Characterization of cell lines bearing the trypanosome (*Tb*) or yeast (*Sc*) *TPI* gene under control of a tetracycline-inducible promoter

TPI activities are shown as means $\pm$ S.D. for at least three measurements, for wild-type trypanosomes and cells bearing the inducible construct, with or without deletion of endogenous *TPI* genes. Results for two half-knockout clones with an inducible gene are shown. Cells were grown in the presence (black bars) or absence (grey bars) of tetracycline. The cells expressing *ScTPI* were harvested 24 h after addition of 250 ng/ml tetracycline.

made a conditional knockout line. Bloodstream trypanosomes expressing the Tn10 *tet* repressor were transfected with a construct bearing the trypanosome *TPI* (*TbTPI*) gene under control of a tetracycline-inducible promoter. In these cells, transcription of the inducible *TPI* gene is inhibited by the *tet* repressor unless tetracycline is present. In the presence of tetracycline, the cells had 2–3-fold the TPI activity of wild-type cells, whereas in the absence of tetracycline, TPI activity was normal (Figure 1). Overexpression of TPI had no effect on parasite growth *in vitro*.

Next, one endogenous copy of *TPI* was replaced by the *NEO* gene. Several cell lines were obtained with this genotype (not shown; [16]); we selected two lines in which the inducible *TPI* gene was not expressed in the absence of tetracycline, so that the level of TPI activity without tetracycline was half the normal level (Figure 1). With tetracycline, TPI activity was about 2–3-fold normal. When extracts of the cells incubated with tetracycline were examined by Western blot, two TPI bands were seen. Results for one line are in Figure 2(A); the other line was similar. The upper band corresponded to the inducible TPI, as it bears a short extension at the C-terminus (see the Materials and methods section). After tetracycline withdrawal, the level of inducible TPI declined so that it was virtually undetectable within 2 days. The reduction of TPI to half the normal level had no effect on trypanosome growth.

We next added tetracycline to the medium, to maintain expression of the inducible copy, and transfected the second replacement construct bearing the *BST* (blebbistatin-resistance) marker. Two independent doubly resistant lines were examined in detail. Southern-blot analysis (not shown; [16]) confirmed that these cells had the genotype *TETR BLE TPI^{tr} HYG $\Delta tpi::NEO/\Delta tpi::BST$* . Thus all TPI protein was now encoded by the inducible gene copy. The effect of tetracycline withdrawal is illustrated for one line in Figure 2; the other line was similar. Initially, as expected, only the inducible TPI was present. After withdrawal of tetracycline, however, the inducible TPI protein and enzyme activity no longer declined to undetectable levels. We measured how much TPI was present by Western blotting and by enzyme assay. Results for different experiments and methods showed that, 2 days after tetracycline withdrawal, levels of TPI ranged between 9 and 22% of the normal level, with an

Figure 2 Characterization of cells bearing the *TPI* gene under control of a tetracycline-inducible promoter

(A) Western blot of trypanosomes extracts at various times after withdrawal of tetracycline. The panel on the top left ('half-KO') shows results from cells bearing an inducible copy and a single endogenous copy of *TPI* (*TPI^{tr} $\Delta tpi::NEO/TPI$*). The panel on the right ('KO') shows the results in cells without any remaining endogenous copies of *TPI* (*TPI^{tr} $\Delta tpi::NEO/\Delta tpi::BST$*). The inducible *TbTPI* bears a short C-terminal extension, so it migrates more slowly than the endogenous copies in the gel. (B) Growth curves of trypanosomes with and without tetracycline. Results for two typical cultures are shown, but these effects have been reproduced many times. Cultures were diluted daily to 4×10^4 cells/ml as required. Closed circles and continuous lines, with tetracycline; open circles and broken lines, without tetracycline. The amounts of TPI (measured by Western blot) relative to control wild-type cells in the cultures without tetracycline are indicated for the first 6 days.

average of about 15%. At 4 days after tetracycline withdrawal, the TPI had begun to increase, to 23–36%. These results suggested that control of *TPI* gene transcription by the *tet* repressor was at least partially lost. We have seen this phenomenon several times previously when dealing with essential genes controlled by this system (see, for example, [26,27]). The results of serial dilutions of the cultures in microtitre plates indicated that as many as half of the *TPI* conditional knockout cells were escaping tetracycline control (results not shown); this is far too frequent to be caused by mutation and suggests that the escape is an epigenetic phenomenon [26,27].

The level of TPI seemed to reach a minimum about 48 h after tetracycline withdrawal. At this point, the cells were clearly surviving, but the growth rate had been halved (Figure 2B), indicating that TPI was limiting for cell growth.

Computer simulation of TPI depletion

At first sight it was surprising to find that TPI is essential for trypanosome growth, since it was expected that glycolysis could proceed in the mutant via equimolar production of glycerol and PYR. To clarify the role of TPI, we used the kinetic model of glycolysis [1,2]. The original model assumed that GPI and TPI were at equilibrium. This model was therefore modified to include the kinetics of both enzymes. We then varied the $V_{\max}$ of TPI to simulate the effects of depleting the enzyme (Figure 3). The model predicts that TPI has a huge overcapacity; only when the TPI activity decreases below 15% should glycerol increase

Figure 3 Computer prediction of the effects of reducing TPI activity on glycolytic flux

(A) The effect of reducing the TPI activity on the steady-state intraglycosomal and cytosolic ATP/ADP ratios and glycosomal DHAP. The 100% and 15% TPI levels are indicated by the broken line. (B) Effect of reducing TPI on the steady-state flux of glucose, PYR and glycerol. (C) Expanded version of (B) showing the region between 12% and zero TPI.

and PYR production decrease as expected according to the 'anaerobic' glycolysis scheme (Figure 3B).

As TPI decreased to below 15% in the simulations, a situation with high flux and equimolar PYR and glycerol production was never reached. Instead, all fluxes (PYR, glycerol and glucose) decreased to zero (Figure 3C). The reason for this is that the

GPO is still active. To maintain the NAD⁺/NADH balance in the glycosome when TPI is inhibited, all glycerol phosphate has to be converted into glycerol. GPO, however, reoxidizes some of it to DHAP, which cannot be reconverted into glycerol phosphate, since that would cause an NADH imbalance in the glycosome. Thus the model shows that DHAP should accumulate in the glycosome when the activity of TPI decreases to below 20% (Figure 3A). This could inhibit glycosomal aldolase. In contrast, using the assumptions of the model, cytosolic [DHAP] is relatively unaffected by reductions of [TPI]. The basal level is about 4 mM, rising to about 4.4 mM at 10% TPI and 5 mM at zero [TPI]. In addition to the effects on glycosomal [DHAP], the model predicts that the glycosomal and cytosolic ATP and ATP/ADP levels will decline drastically at low [TPI] (Figure 3A). At 100% [TPI] the cytosolic and glycosomal ATP levels are about 2.5 mM; at 15% [TPI] the glycosomal [ATP] is already below 1 mM, while cytosolic [ATP] decreases precipitously below 15% [TPI]. These effects on ATP production and the ADP/ATP ratio may partially explain the reduced growth rate.

To see if these predictions were correct, we measured three parameters: PYR production and total cellular ATP and DHAP. It is not possible to prepare organelles of the glycosome/peroxisome family in a non-leaky state, so glycosome internal pools could not be measured. The model predicts that, at 15% [TPI], there should be very little decrease in PYR production. Indeed, PYR production was only very marginally (but reproducibly) lower than normal (results not shown). A very slight decrease in ATP/ADP is predicted also, with a more pronounced effect on the glycosome internal pool. When we measured total ATP levels in the cells, we saw no significant decrease (Table 2). This was not unexpected: as the cell growth rate is halved in the cells with reduced [TPI], the rate of ATP consumption is presumably also diminished. Finally, when the level of TPI is at 10%, a 4-fold increase in glycosomal DHAP is predicted, but the glycosomes occupy 4–5% of the total cell volume, so the predicted increase for the whole cell is only 10%. DHAP was measured in normal cultured trypanosomes and in cultures of the inducible knockout line, either with tetracycline or 2 days after tetracycline withdrawal. The cellular DHAP concentration was calculated using previous estimates of cellular volume and protein content [1]. The results in Table 2 confirmed that TPI depletion results in DHAP accumulation. The basal level of DHAP was, however, unexpectedly low, and the increase after TPI reduction greater than predicted.

The fact that the predictions of the model were qualitatively, but not quantitatively, correct suggested that, at least for trypanosomes cultured *in vitro*, one or two kinetic parameters (such as those for the GPO, or the unknown glycosomal transporters) may have been set incorrectly in the model. Since the kinetics and mechanism of metabolite transport across the glycosomal membrane are unknown, we had to make assump-

Table 2 Effect of TPI depletion on pyruvate production and total cellular DHAP and ATP

The pyruvate production and total cellular DHAP and ATP for TPI-depleted and normal cells are given as the means for at least three independent measurements. Predicted values are also shown.

TPI activity (%) ...	Computer prediction		Measured value	
	100	15	100	15
Pyruvate (nmol · min ⁻¹ · mg of protein ⁻¹)	142	128	208	166
Glycerol (nmol · min ⁻¹ · mg of protein ⁻¹)	6.8	14.6	n.d.	n.d.
Total DHAP (mM)	3.89	4.19	0.16	0.4
Total ATP (mM)	2.76	2.59	0.28	0.27

Figure 4 Effect of expressing cytosolic TPI on trypanosome growth

All growing cultures were diluted daily to 2×10^5 cells/ml as required and grown with or without 250 ng/ml tetracycline. Symbols are as for Figure 3: closed circles and continuous lines, with tetracycline; open circles and broken lines, without tetracycline.

tions about this in the model. To investigate the implications of such assumptions we calculated the effects of TPI depletion if the glycosomal membrane was completely removed and all enzymes were dispersed throughout the cytosol [7]. Qualitatively the results were the same. For example, when the TPI activity decreased to zero, the fluxes of glucose, PYR and glycerol, and the [ATP]/[ADP] ratio also decreased to zero. The predicted DHAP accumulation was drastically elevated, so that at 15% [TPI] the level of DHAP was predicted at 855 mM.

Is compartmentalization of TPI necessary?

The computer modelling suggested that, if trypanosomes can survive by anaerobic glycolysis, it might be possible to rescue a Δtpi strain by preventing DHAP accumulation and restoring the glycosomal redox balance. This could in theory be achieved using a cytosolic TPI, which would convert the DHAP into GA-3-P (Scheme 1, broken lines), which could re-enter the glycosome if an appropriate transporter were present. We attempted this experiment by creating bloodstream trypanosomes that contained a tetracycline-inducible copy of *S. cerevisiae* TPI in addition to the endogenous *TbTPI* genes. Typical results are shown in Figures 1 and 4. At 1 day after the addition of tetracycline, the overall TPI activity was 2–3-fold the wild-type level; we confirmed that the additional TPI was in the cytosol [27a] by digitonin fractionation (not shown; [16]). Figure 4 shows a growth curve for one cell line after tetracycline addition. Expression of *ScTPI* severely inhibited parasite growth; in several other experiments, with this and five other cell lines, at least 90% of the cells died.

Effect of SHAM on trypanosome cultures

An alternative way to rescue cells lacking TPI would be to inhibit the GPO. This should prevent accumulation of DHAP. We therefore first tried incubating normal trypanosomes with SHAM, a chelator which inhibits GPO. We discovered, however, that SHAM kills *in vitro*-cultured bloodstream trypanosomes. The effects on growth were seen for both the 427 and 927 strains, and became visible after about 8 h. The minimum concentration for complete growth inhibition over a 24 h period was only 40 μ M, which is twice the K_i of SHAM for the GPO in homogenates [28]. The toxicity we observed was not due to glycerol accumulation [10], as it was seen even at 100 cells/ml.

When we incubated procyclic trypanosomes, which are not dependent on the GPO, with 250 μ M SHAM there were no effects on cell growth. Since SHAM is an ion chelator, its effects could nevertheless be due to non-specific inhibition of some other bloodstream-trypanosome-specific target.

Is the GPO essential?

The results seen upon SHAM prompted us to re-examine the role of GPO. We tried incubating bloodstream and procyclic trypanosomes overnight under truly anaerobic conditions (ascorbate oxidase and ascorbate to deplete dissolved oxygen, with an anaerobic atmosphere, see the Materials and methods section). Both cultures died, but the specificity of this effect is difficult to determine. We therefore decided to try to knockout the GPO activity. GPO is composed of a flavoprotein (GDH) and an alternative oxidase (TAO). We targeted the TAO component, as the sequence is available [17]. Plasmids were constructed in order to replace the *TAO* gene with the *NEO* or *HYG* markers (see the Materials and methods section). Unfortunately no transformants at all were obtained with either plasmid, although control experiments confirmed that the design of the constructs was appropriate. The *HYG*-containing knockout plasmid was able to confer hygromycin resistance when we linearized it within the plasmid backbone and targeted it into a plasmid DNA which had been previously integrated at the tubulin locus, so it is possible that insufficient mRNA was made from the *TAO* locus to allow selection of drug-resistant trypanosomes.

As an alternative, we generated trypanosomes expressing a double-stranded (ds) RNA corresponding to the first 816 nt of the *TAO* gene, again under control of the tetracycline-inducible promoter (see the Materials and methods section). Expression of dsRNA in trypanosomes results in highly sequence-specific destruction of the homologous mRNA [18,20,29]. If the targeted gene is not essential, the expression of the protein product can often be reduced to less than 5% of wild-type levels ([18,20,29]; M. Drozd and C. Clayton, unpublished work). Five clones of bloodstream trypanosomes containing the construct were obtained. We show here the results obtained with the clone that showed the greatest inducible reduction of *TAO* mRNA levels. After 48 h in the presence of *TAO* dsRNA, the corresponding mRNA had virtually disappeared (Figure 5A). Induction of *TAO*-targeted dsRNA expression reduced the growth rate (Figure 5B): the average doubling time increased from 7.4 to 11.8 h, and the cells were killed by addition of 1 mM glycerol, as expected. We have no antibody to TAO, so we assessed the effect on aerobic metabolism directly, by measuring oxygen consumption of intact trypanosomes. In the absence of tetracycline (and therefore dsRNA), the consumption was 106 nmol of O_2 /min per 6.5×10^7 cells (mean of two measurements), which was similar to the level in wild-type trypanosomes. At 48 h after induction of the dsRNA, the oxygen consumption was 24 nmol of O_2 /min per 6.5×10^7 cells (mean of two measurements). Addition of SHAM completely inhibited oxygen consumption in the controls, and eliminated residual consumption in the mutants, as expected.

To assess the relationship between oxygen consumption and GPO activity, we again turned to the computer model. From the calculations (not shown), TAO has a considerable overcapacity. A reduction of 50% in the V_{max} is predicted to have almost no effect on the oxygen consumption and the ATP/ADP ratio. The reduction in oxygen consumption to 22% corresponds in the model to a reduction in [TAO] to about 4.5% of the wild-type level, which is consistent with the reduction we saw in *TAO* mRNA. At this point, ATP consumption and production are

Figure 5 Inhibition of TAO using inducible dsRNA

(A) Effect of dsRNA induction on the level of TAO mRNA. (B) Growth of TAO-deficient cells. The experiment was done as in Figure 4, except that cultures were diluted daily to 2.5×10^5 cells/ml as required and grown with or without 100 ng/ml tetracycline and/or 1 mM glycerol. Symbols are: closed circles, with tetracycline, TAO-deficient; closed squares, TAO-deficient with glycerol; open circles, without tetracycline, normal TAO; open squares, normal TAO with glycerol.

predicted to be reduced by 66%. Thus the metabolic results obtained were consistent with the decreased growth rate.

DISCUSSION

This paper describes the integration of *in vivo* experimentation and computer modelling to determine the roles of TPI and TAO in trypanosomes. The results suggest that trypanosomes are obligate aerobes that require TPI for survival. For optimal growth more than 15% of the wild-type TPI enzyme level, and more than 5% of normal TAO activity, are required. In addition, the expression of TPI in the cytosol inhibited parasite growth.

In vitro experiments on microbody metabolite transporters are virtually precluded by the susceptibility of the organelles to damage during isolation, so that the most reliable information is obtained by reverse genetic approaches. Such experiments have demonstrated that the peroxisomes of *S. cerevisiae* are impermeable to acetyl-CoA and NAD(H) [8]. The metabolic pathways observed for trypanosome glycosomes also suggest that NAD(H) cannot cross the glycosomal membrane [5]. The deleterious effects of expression of cytosolic TPI are consistent with this. ScTPI in the trypanosome cytosol should convert cytosolic DHAP into GA-3-P. Unless this were rapidly re-imported (presumably via the DHAP/glycerophosphate exchanger), it would be converted into 1,3-BPGA as trypanosomes have a cytosolic isoenzyme of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (see the broken lines in Scheme 1). Whether or not the 1,3-BPGA can be re-imported, gradual depletion of NADH inside the glycosome is predicted. The results therefore suggest that GA-3-P is poorly transported into glycosomes. Deletion of the cytosolic GAPDH genes (if not lethal) might allow further analysis of transporter specificity.

There are two possible explanations for why trypanosomes require TPI. In the absence of the enzyme, the model predicts that ATP cannot be produced at all. This would of course be lethal. In addition, at low [TPI] the accumulating DHAP in the

glycosome could inhibit aldolase, or might be converted spontaneously to methylglyoxal, which would be toxic to the parasites. A 30–40-fold increase in DHAP was observed in TPI-deficient human erythrocytes [30]. It has been reported that a TPI mutant of *S. cerevisiae* is unable to grow on high levels of glucose and also accumulates DHAP [31], but the complete knockout is lethal [32].

An alternative (or additional) explanation for a TPI requirement is that trypanosomes cannot survive by completely anaerobic metabolism, with a yield of one ATP molecule per glucose molecule. Pharmacological evidence suggested that this might be the case, as the effects of SHAM were consistent with a dependence on aerobic metabolism. These results were not inconsistent with the results concerning SHAM in the literature, as neither the *in vivo* nor the *in vitro* studies documented exposure of the parasites to levels of active inhibitor exceeding the K_i for more than 7 h [10,33–36]. Another GPO inhibitor, ascofuranone, was again cytotoxic only in the presence of glycerol [12,37], but the action of this drug is strongly inhibited by serum albumin [12], which could compromise its action in prolonged incubations. We have been unable to find any published pharmacokinetic data on ascofuranone and have also not been able to obtain a sample.

Our attempts to knockout even one copy of *TAO* failed completely, but there must have been technical reasons for this as we were able to achieve a very substantial reduction in *TAO* mRNA using inducible RNA interference. The reduction we obtained, in our experience, approaches the maximum that can be obtained using either RNA interference or the inducible knockout strategy employed for TPI. We cannot therefore tell, with available technology, whether the TAO is essential or not. Our results did, however, demonstrate that significant growth inhibition is seen when TAO activity is reduced so as to halve the oxygen consumption. If the TAO is essential, any inhibitory drug would have to inhibit TAO by considerably more than 95% in order to have effective cytostatic activity.

With the completion of many whole genome sequences, there is an increasing emphasis on proteomic approaches for the description of metabolic capabilities of organisms and cells. The use of computer modelling is an integral part of this process. The work described here demonstrates the effectiveness of combining biochemical, genetic and theoretical approaches in the study of metabolism.

We thank Professor M. Stitt (Department of Botany, University of Heidelberg, Heidelberg, Germany) and members of his laboratory for use of spectrophotometer, Dietmar Steverding (Department of Tropical Medicine and Parasitology, University of Heidelberg, Heidelberg, Germany) for help with, and use of, the oxygen electrode, Claudia Hartmann (ZMBH) for cloning the yeast *TPI* gene and making the *ScTPI* inducible cell lines, and Drifa Gudjonsdottir-Plank for technical support. Yeast genomic DNA was supplied by the laboratory of R. Jansen (ZMBH). We thank Elisabetta Ullu (Yale University, New Haven, CT, U.S.A.), Elisabeth Wirtz and George Cross (Rockefeller University, New York, NY, U.S.A.) and Paul Englund (The Johns Hopkins University, Baltimore, MD, U.S.A.) for gifts of plasmids. Professor A. Tielens suggested we should test anaerobic conditions. This work was supported by the BMBF (Bundesministerium für Bildung und Forschung) Schwerpunkt 'Tropenmedizin in Heidelberg' and by the DFG (Deutsche Forschungsgemeinschaft) (SFB544, Kontrolle tropischer Infektionskrankheiten).

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Received 11 October 2000/23 February 2001; accepted 6 April 2001