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Compartmentation of phosphoglycerate kinase in *Trypanosoma brucei* plays a critical role in parasite energy metabolism

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ABSTRACT **African trypanosomes compartmentalize glycolysis in a microbody, the glycosome. When growing in the mammalian bloodstream, trypanosomes contain only a rudimentary mitochondrion, and the first seven glycolytic enzymes, including phosphoglycerate kinase, are located in the glycosome. Procyclic trypanosomes, growing in the gut of tsetse flies, possess a fully developed mitochondrion that is active in oxidative phosphorylation. The first six glycolytic enzymes are still glycosomal, but phosphoglycerate kinase is now found in the cytosol. We demonstrate here that bloodstream trypanosomes are killed by expression of cytosolic phosphoglycerate kinase. The toxicity depends on both enzyme activity and cytosolic location. One possible explanation is that cytosolic phosphoglycerate kinase creates an ATP-generating shunt in the cytosol, thus preventing full ATP regeneration in the glycosome and ultimately inhibiting the first, ATP-consuming, steps of glycolysis.**

All members of the order Kinetoplastida contain microbodies harboring glycolytic enzymes (1). Some enzymes are exclusive to the glycosome, whereas others are present in both glycosome and cytosol. In addition, the compartmentalization is developmentally regulated to varying degrees depending on the species. Kinetoplastid metabolism is simplest in the African trypanosome *Trypanosoma brucei* (2). *T. brucei* multiplies extracellularly as "bloodstream forms" in the blood and tissue fluids of mammals, and as "procyclic forms" in the gut of the tsetse fly vector. The bloodstream forms possess only a rudimentary mitochondrion and survive exclusively by substrate-level phosphorylation, with glucose— abundantly available in the environment— as the only energy source. In tsetse flies, in which amino acids are the predominant substrate; the mitochondrion is well developed, with citric acid cycle enzymes and a respiratory chain (2).

One of the many enzymatic differences between bloodstream and procyclic forms is the location of phosphoglycerate kinase (PGK). *T. brucei* has three *PGK* genes (3, 4). One, *PGKA*, encodes a minor glycosomal variant (PGKA) that is expressed at low levels in both bloodstream and procyclic forms (5, 6). The second gene, *PGKB*, encodes the major cytosolic enzyme PGKB, which is present only in procyclic forms. The third gene, *PGKC*, encodes the major glycosomal enzyme PGKC, expressed only in bloodstream forms (3). PGKC is directed to the glycosome by a signal sequence present at the end of a 20-amino acid C-terminal extension (7–9). The developmental regulation of *PGKB* and *PGKC* expression is mediated posttranscriptionally by sequences in the 3′ untranslated regions of the mRNAs (10). Here we describe experiments showing that correct developmentally regulated compartmentalization of PGK is vital for bloodstream trypanosome survival.

MATERIALS AND METHODS

Plasmid Constructs. Plasmids for inducible expression of *PGK* genes were constructed by replacing the chloramphenicol acetyltransferase or luciferase cassettes in pHD 615, pHD 616, or pHD 451 (11) with the gene of interest. Details of the vectors can be found at http://www.zmbh.uni-heidelberg.de. The *PGKB* and *PGKC* gene cloning and mutagenesis were all done by using PCR, and reconstructed sequences are available from the authors. All plasmids used are listed in Table 1.

Trypanosome Culture and Transfection. Trypanosomes expressing the *Tet* repressor (from plasmid pHD 449 integrated at the tubulin locus) were transfected with *Not*I-linearized inducible plasmids, and transformants were selected in the absence of tetracycline as described (11). Cloning was done by limiting dilution. To induce expression of the added *PGK* gene, tetracycline (up to 5 μg/ml) was added. Cells were cultured in HMI-9 in plastic flasks in an atmosphere of 5% CO_2. Volumes of 100–200 ml were placed in 1- to 2-liter flasks that were equilibrated with 5% CO_2, then closed and placed on a roller overnight.

To measure pyruvate production, cells were washed three times in RPMI medium 1640 then resuspended in RPMI 1640 supplemented with L-glutamine (300 mg/liter), $NaHCO_3$ (2 g/liter), Hepes (25 mM), glucose (to 4.5 g/liter), bathocuproinedisulfonate (50 μM), hypoxanthine (1 mM), thymidine (160 μM), $CaCl_2{\cdot}2H_2O$ (219 mg/liter), L-alanine (25 mg/liter), cysteine (182 mg/liter), 2-mercaptoethanol (14 μl/liter), and 20% dialyzed fetal calf serum. This medium supports growth of the parasites overnight almost as well as HMI-9.

Cell Fractionation. Digitonin fractionation of trypanosomes was performed as described (12); trypanosomes (10^7) in 450 μl of homogenization buffer [25 mM Tris·Cl, pH 7.8/1 mM EDTA/10% (wt/vol) sucrose/2 μg/ml leupeptin] were treated for 2 min at 37°C with 0.6 mg of digitonin per mg of protein. Glycolytic enzymes were assayed as described (13) and Western blots were prepared with trichloroacetic acid (TCA)-precipitated protein by using enhanced chemiluminescence (12).

Metabolite Concentrations. The concentrations of glycolytic intermediates and ATP in trypanosome extracts (14) were measured by enzyme assays (15) using a two-wavelength spectrophotometer (Sigma ZFP-22, Eppendorf; measurement wavelength 334 nm, reference wavelength 405 nm). Metabolites measured were glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, phosphoenolpyruvate, and pyruvate.

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Abbreviations: PGK, phosphogycerate kinase; TCA, trichloroacetic acid; UTR, untranslated region.
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Table 1. List of plasmids used in this study

pHD no.	Vector	Insert	3′ UTR
531	pHD 451	PGKB	Δ*ALD*
532	pHD 451	PGKC	Δ*ALD*
735	pHD 615	PGKB	*VSG*
736	pHD 615	PGKC	*VSG*
737	pHD 615	PGKB His-393 → Lys	*VSG*
738	pHD 615	PGKB His-393 → Glu	*VSG*
739	pHD 615	PGKB His-393 → STOP	*VSG*
740	pHD 616	PGKB	*ACT*
741	pHD 616	PGKC	*ACT*
742	pHD 616	PGKB His-393 → Lys	*ACT*
743	pHD 616	PGKB His-393 → Glu	*ACT*
744	pHD 616	PGKB His-393 → STOP	*ACT*
777	pHD 615	PGKCΔRWSSL	*VSG*
778	pHD 616	PGKCΔRWSSL	*ACT*
779	pHD 451	PGKB His-393 → Lys	Δ*ALD*
780	pHD 451	PGKB His-393 → Glu	Δ*ALD*
781	pHD 451	PGKB His-393 → STOP	Δ*ALD*

Details of the vectors are to be found in ref. 11.

RESULTS

Inducible Expression of Cytosolic PGK in Bloodstream Trypanosomes Causes Cell Death. During attempts to replace glycosomal PGK with cytosolic PGK in bloodstream trypanosomes, we were unable to obtain any clones expressing cytosolic PGK. To investigate whether cytosolic PGK was toxic, we generated bloodstream trypanosomes that contained a *PGKB* coding region whose expression was mediated by a tetracycline-inducible promoter. The construct used to generate the cell lines contained a constitutively expressed hygromycin resistance cassette downstream of the *PGKB* coding region (11), allowing selection of transformants. After transfection into trypanosomes expressing the *Tet* repressor, hygromycin-resistant transformants were selected in the absence of tetracycline, so that the inducible promoter was switched off. Under these conditions, cells (Fig. 1) grew as well as control cells, which expressed repressor but lacked an inducible gene (Fig. 1). (These cells were used as controls throughout subsequent experiments.) However, when tetracycline was added to the medium, the cells with the inducible cytosolic PGK (*PGKB* gene) stopped growing and died (Fig. 1). This confirmed that expression of PGKB in bloodstream trypanosomes is toxic.

As expected, the addition of tetracycline had no effect on growth of cells lacking an inducible gene (Fig. 1). As another control, we included cells with an inducible glycosomal *PGKC* gene. Their growth also was unaffected by the addition of tetracycline (Fig. 1).

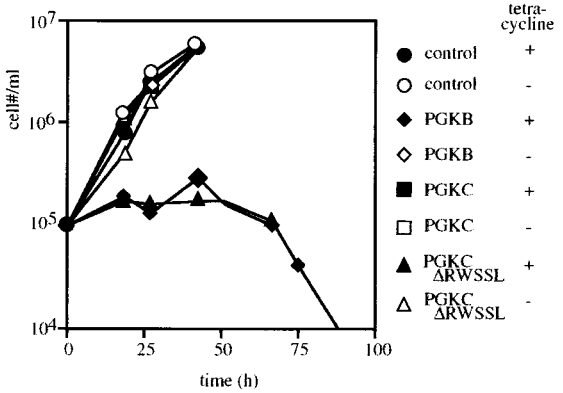

FIG. 1. Expression of cytosolic PGK in bloodstream trypanosomes is toxic. Trypanosomes expressing the *Tet* repressor and containing integrated copies of plasmids pHD 735,736, or 777 were grown in the presence or absence of tetracycline (5 μg/ml). All cultures were started at 10^5 cells per ml. The nature of the inducible gene and the presence or absence of tetracycline in the medium for each symbol are indicated on the right.

Toxicity Depends on the Level of PGKB Expression. To investigate the relationship between the level of PGKB expression and toxicity, we grew cells containing the inducible gene in various concentrations of tetracycline. The results of this experiment are shown in Fig. 2. Cells were cultured for 24 h in the presence of tetracycline, then fractionated with digitonin to separate the cytosol from the glycosomal pellet. In the absence of tetracycline, most of the PGK was in the pellet fraction. (Perfect fractionation was not possible, so 10–20% cross-contamination of fractions was always seen.) As the amount of tetracycline was increased, a band corresponding to PGKB, which is slightly smaller than PGKC because of the absence of the C-terminal extension, became detectable in the supernatant fraction. At 1 ng/ml tetracycline, PGKB was just detectable by Western blot, and growth inhibition was discernible. This level of expression is too low to be quantified accurately. With 5 ng/ml and 10 ng/ml tetracycline, growth was completely inhibited, although PGKB constituted less than 5% of total PGK activity in the cells.

When we cultivated trypanosomes containing inducible PGKB in the presence of high levels of tetracycline, we sometimes found that instead of dying, the cell population resumed growth after a few days. The newly growing cells seemed to have escaped from tetracycline control because they no longer expressed PGKB. We have not yet investigated this phenomenon in detail; it may be a consequence of mutations either in the inducible gene or in the inducible expression system. Trypanosomes are known to exhibit a high mutation rate when placed under negative selection (16).

The Location of PGK Is Critical. The cells used for the dose-response curve (Fig. 2*b*) contained a construct in which the inducible *PGK* coding region was followed by a truncated 3′ untranslated region (UTR) from the aldolase locus (Δ*ALD*). This results in rather low expression of the gene in bloodstream trypanosomes. In most experiments with bloodstream trypanosomes we therefore used constructs in which the gene to be expressed was followed by a variant surface glycoprotein (VSG) 3′ UTR (Table 1). Fig. 3 shows the results of cell fractionations for bloodstream trypanosomes expressing different PGKs from

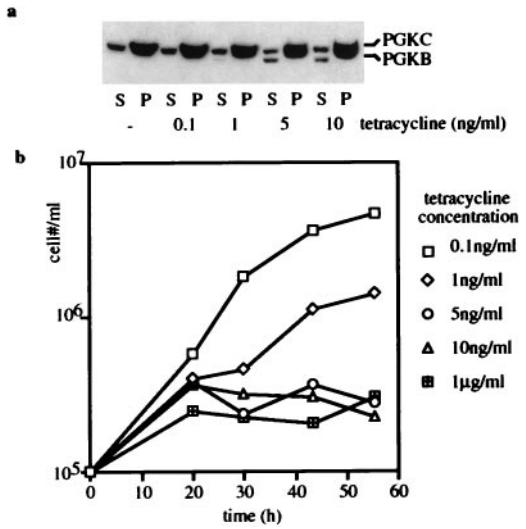

FIG. 2. The toxicity of cytosolic PGK is dose-dependent. Bloodstream trypanosomes containing the *Tet* repressor and pHD 531 (encoding PGKB) were treated with various doses of tetracycline. (*a*) Digitonin was used to fractionate cells (10^7) 24 h after tetracycline addition, and PGK was detected by Western blot using a polyclonal antibody to glycosomal PGK, which also detects the cytosolic enzyme. S, supernatant; P, pellet. (*b*) Growth curves.

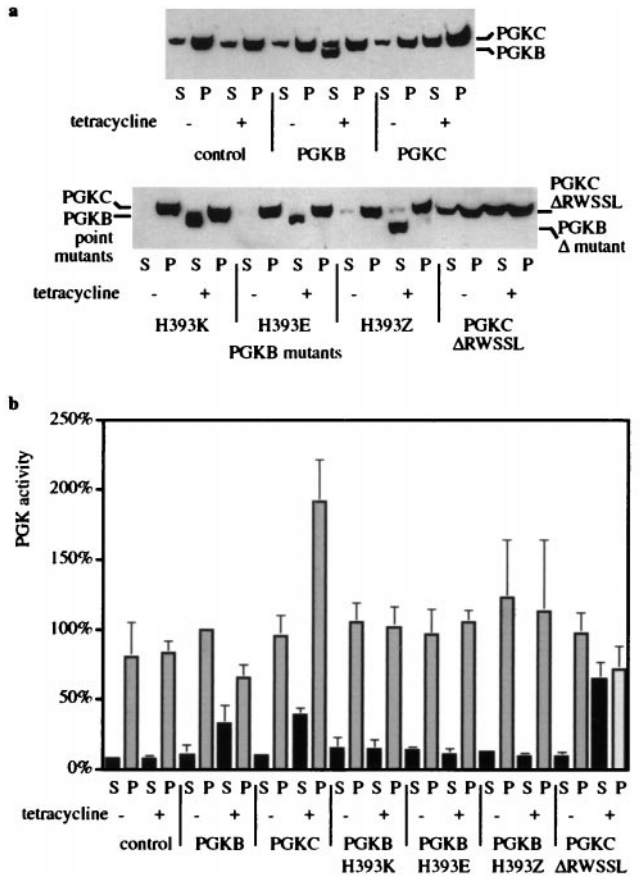

FIG. 3. Location (*a*) and activity (*b*) of various versions of PGK inducibly expressed in bloodstream forms. Cultures (30 ml) initiated at 5×10^5 cells/ml in the presence or absence of 5 μg/ml tetracycline were grown for 24 h; aliquots of 10^7 cells were then subjected to cell fractionation with digitonin. S, supernatant; P, pellet. (*a*) Western blot of TCA-precipitated protein using anti-PGK antibody. (*b*) Enzyme activities were measured from fractions equivalent to 4×10^6 cells. Results were calculated relative to the pellet fraction of the PGKB cells without tetracycline, set to 100%, and are the mean ± standard deviation from three or four independent experiments. Cell lines contained the *PGK* coding regions followed by the *VSG* 3′ UTR.

these constructs. As before, most of the PGK protein (Fig. 3*a*) and enzyme activity (Fig. 3*b*) in control cells was found in the pellet fraction (glycosomes), and addition of tetracycline had no effect. After addition of tetracycline to cells containing an inducible copy of *PGKB*, PGKB was detected by Western blot (Fig. 3*a*) and an increase in overall PGK activity was observed. About one-third of the activity was now found in the cytosol (Fig. 3*b*). Induction of PGKC expression resulted in increased activity in the glycosomes as expected. (Although the activity in the cytosol also appears increased, this is probably because of leakage of the organelles during fractionation.)

The main difference between cytosolic PGK and glycosomal PGK is the presence of a 20-amino acid C-terminal extension in glycosomal PGK. However, there are also internal sequence differences. To ensure that the toxicity of cytosolic PGK was not caused by these internal differences, we expressed in bloodstream trypanosomes a version of PGKC lacking the final 5 amino acids, which are responsible for glycosomal targeting. This mutant PGKC, PGKCΔRWSSL, was enzymatically active, and located in the cytosol (Fig. 3); its expression also was toxic (Fig. 1). This demonstrates that the cytosolic location, not the precise details of the amino acid sequence, is important for toxicity.

Enzyme Activity Is Required for Toxicity. We anticipated that the toxicity of cytosolic PGK was a consequence of a metabolic imbalance created by enzyme activity in an inappropriate location. However, it could have been a nonspecific consequence of cytosolic protein accumulation. To test the requirement for enzyme activity, we generated mutant versions of *PGKB* in which the histidine 393 codon had been replaced by a stop codon or by codons for lysine or glutamic acid. Replacement of the equivalent residue in the *Saccharomyces cerevisiae* enzyme, histidine-388, by glutamine reduces the K_m for ATP threefold, the k_{cat} of the enzyme is decreased fivefold (17), probably because interdomain movement during catalysis is affected (18). The introduction of a stop codon truncates the enzyme by 27 amino acids. A 15-amino acid C-terminal deletion of yeast PGK reduced activity to 1% and compromised enzyme stability (19).

To test the activity of the mutant trypanosome enzymes, we first expressed them in procyclic trypanosomes, because a higher level of inducible expression is obtained in this life cycle stage than in the bloodstream forms. Results are shown in Fig. 4. Cells lacking an inducible gene, or cells in the absence of tetracycline, showed partitioning of PGK into the cytosolic fraction as expected (Fig. 4*a*). Cells containing an induced copy of PGKB

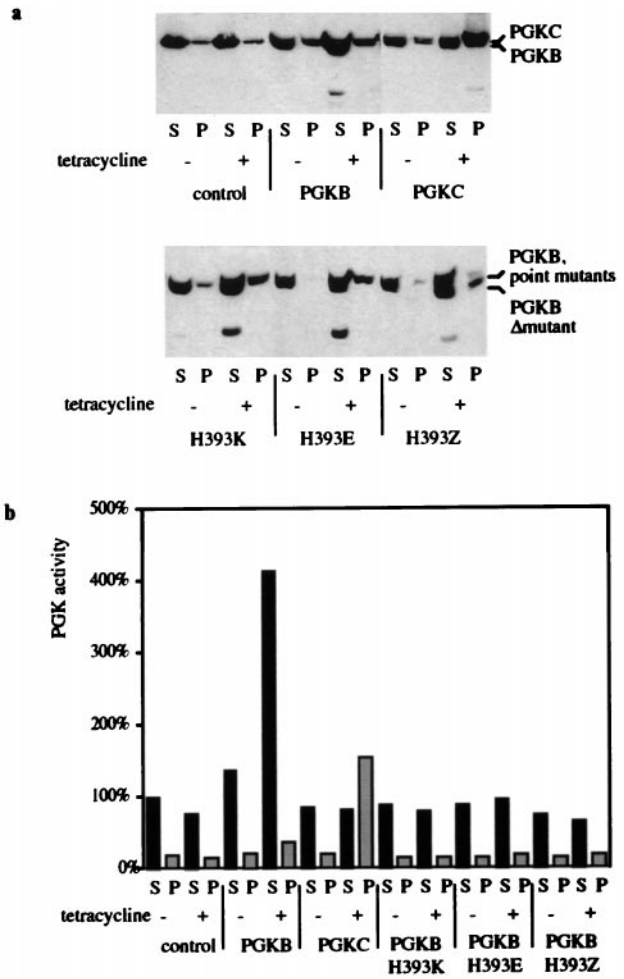

FIG. 4. Location (*a*) and activity (*b*) of various versions of PGK inducibly expressed in procyclic forms. Cultures (10 ml) initiated at 2×10^5 cells/ml in the presence or absence of 5 μg/ml tetracycline were grown for 48 h; aliquots of 10^7 cells were then subjected to cell fractionation with digitonin. S, supernatant; P, pellet. The PGKC cell line was initiated at 9×10^5 cells/ml because the cells grow slightly more slowly. (*a*) Western blot of TCA-precipitated protein using anti-PGKC antibody. (*b*) Enzyme activities were measured for fractions equivalent to 2×10^6 cells. Results were calculated relative to the supernatant fraction of the control cells without tetracycline, set to 100%, and are the mean from two independent experiments. Cell lines contained different inducible *PGK* coding regions followed by the *ACT* 3′ UTR.

exhibited 4 times higher activity in the cytosol than in control cells (Fig. 4*b*); there was no detectable alteration in phenotype. (In these overexpressing cells, some PGKB contaminates the pellet fraction.) In contrast, cells with induced PGKC showed a doubling of PGK activity, with all of the new activity confined to the glycosome (Fig. 4*b*); this caused a decrease in the growth rate such that the doubling time was increased by about 10% (not shown). In cells expressing the mutant versions of PGKB, protein was clearly produced (Fig. 4*a*) but no additional enzyme activity was detected (Fig. 4*b*). All cells that overproduced PGK showed some degradation of the protein, with possibly more degradation in the mutant versions (Fig. 4*a*).

The mutated enzymes were now expressed in bloodstream trypanosomes. Cell fractionations (Fig. 3*a*) showed that each protein was produced and located in the cytosol. As expected, no increase in enzyme activity was detected, and we observed no change in phenotype.

Glycolytic Intermediates. We concluded from these experiments that the toxic effect of cytosolic PGK in bloodstream forms depended on enzyme activity, although the levels of enzyme required for toxicity were very low. We next compared the amounts of six intermediates and ATP in PGKB-expressing and control trypanosomes, both without tetracycline and 24 h after tetracycline addition. All measurements (see *Materials and Methods*) gave results similar to those previously observed (14), and no significant differences were detected between the two cultures. Cells were also removed 24 h after tetracycline addition, and pyruvate production was measured at 37°C over an 8-h period. The medium used for this assay was RPMI 1640 supplemented so as to support trypanosome growth for at least 24 h (see *Materials and Methods*). The rate of pyruvate production by growing cells at a density of 2×10^6 cells/ml was approximately 6 μmol/h per 10^8 cells, as previously reported (20). No difference was seen between the cells expressing PGKB and the controls.

DISCUSSION

The results described here show that the expression of PGK in the cytosol of bloodstream trypanosomes is toxic. The toxicity depends on enzyme activity, implying that it is a consequence of metabolic imbalance between different subcellular compartments. For such an imbalance to arise, there must be effective compartmentation of either substrates or products of the PGK reaction. This in turn implies that the glycosomal membrane is a significant permeability barrier for at least some glycolytic intermediates or products.

Very little is currently known about the transport properties of the glycosomal membrane. Isolated glycosome (and peroxisome) preparations contain a significant proportion of leaky organelles that compromise permeability measurements. However, some conclusions can be drawn from the known metabolic pathways and enzyme compartmentation (21). Bloodstream-form glycosomes must be able to take up glucose and export 3-phosphoglycerate (GLC and 3PG in Fig. 5). In addition, export of glycerol 3-phosphate (G3P) and import of dihydroxyacetone phosphate (DHAP) and phosphate are required (Fig. 5). In contrast, the currently accepted version of bloodstream-form glycolysis reveals no necessity for transport of ATP, ADP, or NAD(H). Impermeability of the glycosomal membrane to NAD(H) would be consistent with results of genetic experiments in *S. cerevisiae* which demonstrated that the membrane of peroxisomes is impermeable to NAD(H) and acetyl-CoA under *in vivo* conditions (22). Impermeability to ATP and ADP also seems plausible. In procyclic *T. brucei*, in which PGK is cytosolic, and in bloodstream *Trypanosoma congolense*, which also have mostly cytosolic PGK (23), ATP can be regenerated within the glycosome during conversion of phosphoenolpyruvate to malate (reviewed in ref. 2).

For cytosolic PGK activity to have detrimental effects, an appropriate substrate must be available within the cytosol. This

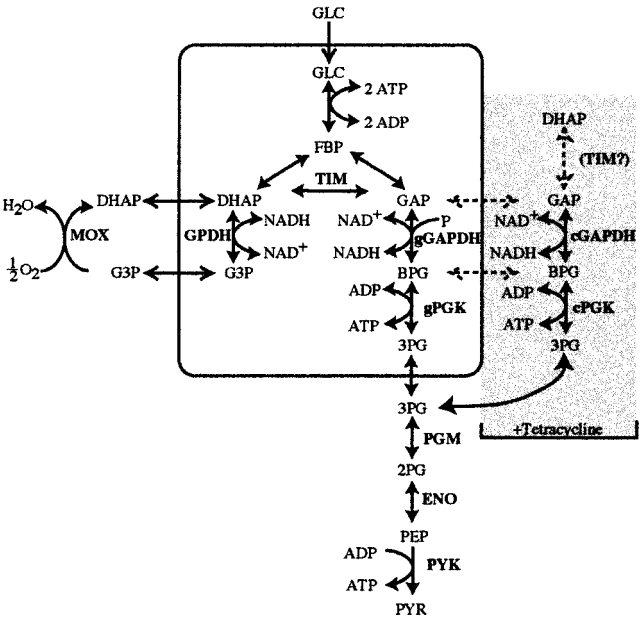

FIG. 5. Possible explanations of PGKB toxicity. The pathway of glucose metabolism in wild-type bloodstream trypanosomes is shown; the additional steps that may be involved in toxicity are in the shaded area on the right. Hypothetical pathways or steps are indicated with broken lines; a hypothetical cytosolic triose-phosphate isomerase is shown as (TIM) in right part of figure. GLC, glucose; FBP, fructose bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; GAP, glyceraldehyde-3-phosphate; BPG, bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; TIM, triose-phosphate isomerase; GPDH, glycerol 3-phosphate dehydrogenase; MOX, mitochondrial oxidase; cGAPDH, glyceraldehyde 3-phosphate dehydrogenase, cytosolic isozyme; gGAPDH, glyceraldehyde-3-phosphate dehydrogenase, glycosomal isozyme; gPGK, phosphoglycerate kinase, glycosomal isozyme; cPGK, phosphoglycerate kinase, cytosolic isozyme; PGM, phosphoglucomutase; ENO, enolase; PYK, pyruvate kinase.

could be either 1,3-bisphosphoglycerate (BPG in Fig. 5), for the ATP-generating direction, or 3-phosphoglycerate (3PG in Fig. 5) for the reverse reaction. These could come either from direct leakage or from conversion of precursors (such as dihydroxyacetone phosphate) by cytosolic enzymes. The existence of cytosolic glyceraldehyde-phosphate dehydrogenase in bloodstream trypanosomes is well established (24). Although the results of cell-fractionation studies place all other enzyme activities from hexokinase to PGK within the glycosome (see, for example refs. 12, 13, and 25; reviewed in ref. 26) it is conceivable that a fraction is in the cytosol, as seen for triose-phosphate isomerase in some *Leishmania* species (27–29). In addition, one study suggested the existence of glycolytic intermediates in the cytosol (14). After feeding trypanosomes with radioactive glucose, the labeling of glycolytic intermediates showed biphasic kinetics, suggesting the presence of two pools—one (including the end-product, pyruvate) that was 50% labeled in 15 sec, and another (70–80% of the total) that equilibrated much more slowly. The authors concluded that the rapidly labeled pool was glycosomal and the rest was cytosolic, and argued that the two pools are in equilibrium (14, 21). Although the activity of the pentose phosphate pathway is very low in bloodstream trypanosomes (30), some contribution to the overall energy balance and metabolite pools cannot be ruled out.

Even given an adequate source of substrate for cytosolic PGK, why should the activity be toxic? Any explanation must take into account the fact that the presence of cytosolic PGK does not appear to affect either pyruvate production or the overall levels of six glycolytic intermediates and ATP. Although this does not exclude the possibility that the distribu-

tion of metabolites has changed (glycosomes occupy only about 5% of the cell volume), there is evidence that the two pools are in equilibrium (see above). We therefore suggest that the cytosolic enzyme competes with the glycosomal enzyme for substrate, as shown in Fig. 5 (broken lines). This would in theory generate additional ATP in the cytosol, but would decrease the amount of ATP generated in the glycosome. The cytosolic and glycosomal pools of ATP and ADP are probably not in equilibrium (31). If the loss of ATP cannot be compensated for by import, an imbalance would be created within the glycosome, which would be depleted of the ATP required for the first two kinase steps.

Although our results do not tell us why kinetoplastids compartmentalize glycolysis within the glycosome, they do show that this compartmentalization can play a vital role in maintaining their energy supply. Moreover, the fact that inappropriate expression of a low level of a single metabolic enzyme can kill bloodstream trypanosomes shows that the strict developmental regulation of trypanosome gene expression is absolutely essential for parasite survival.

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