Contribution of Pyruvate Phosphate Dikinase in the Maintenance of the Glycosomal ATP/ADP Balance in the Trypanosoma brucei Procyclic Form*

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Background: The role of pyruvate phosphate dikinase (PPDK), which catalyzes a reversible reaction, is unknown in many eukaryotes.

Results: Deletion of the trypanosomal PPDK gene affects glycolysis.

Conclusion: In trypanosomes, PPDK works in the glycolytic direction and participates in the maintenance of the glycosomal ATP/ADP balance.

Significance: The glycosomal PPDK provides a metabolic flexibility by producing 2 ATP per phosphoenolpyruvate consumed.

Trypanosoma brucei belongs to a group of protists that sequester the first six or seven glycolytic steps inside specialized peroxisomes, named glycosomes. Because of the glycosomal membrane impermeability to nucleotides, ATP molecules consumed by the first glycolytic steps need to be regenerated in the glycosomes by kinases, such as phosphoenolpyruvate carboxykinase (PEPCK). The glycosomal pyruvate phosphate dikinase (PPDK), which reversibly converts phosphoenolpyruvate into pyruvate, could also be involved in this process. To address this question, we analyzed the metabolism of the main carbon sources used by the procyclic trypanosomes (glucose, proline, and threonine) after deletion of the PPDK gene in the wild-type (Δppdk) and PEPCK null (Δppdk/Δpepck) backgrounds. The rate of acetate production from glucose is 30% reduced in the Δppdk mutant, whereas threonine-derived acetate production is not affected, showing that PPDK function in the glycolytic direction with production of ATP in the glycosomes. The Δppdk/Δpepck mutant incubated in glucose as the only carbon source showed a 3.8-fold reduction of the glycolytic rate compared with the Δpepck mutant, as a consequence of the imbalanced glycosomal ATP/ADP ratio. The role of PPDK in maintenance of the ATP/ADP balance was confirmed by expressing the glycosomal phosphoglycerate kinase (PGKC) in the Δppdk/Δpepck cell line, which restored the glycolytic flux. We also observed that expression of PGKC is lethal for procyclic trypanosomes, as a consequence of ATP depletion, due to glycosomal relocation of cytosolic ATP production. This illustrates the key roles played by glycosomal and cytosolic kinases, including PPDK, to maintain the cellular ATP/ADP homeostasis.

Pyruvate phosphate dikinase (PPDK)3 (EC 2.7.9.1) is an inorganic pyrophosphate (PPi)-dependent enzyme, which reversibly catalyzes conversion of phosphoenolpyruvate (P-enolpyruvate), PPi, and AMP into pyruvate, inorganic phosphate, and ATP. In C4 plants, PPDK operates in the gluconeogenic direction (production of P-enolpyruvate from pyruvate) and contributes to CO2 fixation through photosynthesis. Involvement of PPDK in glycolysis (production of pyruvate from P-enolpyruvate) has been suggested in a number of eukaryotes, including Phytophthora (1), Giardia (2, 3), Entamoeba (4), and Trypanosoma (5–7), however, its coexistence with the ATP-dependent glycolytic pyruvate kinase (EC 2.7.1.40) makes it difficult to address its role. Here we address this question in Trypanosoma brucei, using very powerful reverse genetic approaches developed in this eukaryotic model.

T. brucei is a unicellular eukaryote, belonging to the protozoan order Kinetoplastida that causes sleeping sickness in humans (8). This parasite possesses a complex life cycle during transmission from the bloodstream of a mammalian host (bloodstream stages of the parasite) to the alimentary tract (procyclic stage) and salivary glands (epimastigote and metacyclic stages) of a blood-feeding insect vector, the tsetse fly. The procyclic insect stage of T. brucei, our experimental model in this analysis, develops an elaborate energy metabolism based on different carbon sources, including glucose, proline, and threonine (9–11). Although proline is the major component of the hemolymph of the fly (12), the parasite prefers glucose when this carbon source is available (13, 14).

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3 The abbreviations used are: PPDK, pyruvate phosphate dikinase; PEPCK, phosphoenolpyruvate carboxykinase; PGKB, cytosolic phosphoglycerate kinase isoform; PGKC, glycosomal phosphoglycerate kinase isoform; TDH, threonine 3-dehydrogenase; i and ni, tetracycline-induced and uninduced, respectively; rPGKC, recombinant glycosomal PGKC isoform; GK, glycerol kinase; BSD, blasticidin; PAC, puromycin.

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The procyclic trypanosomes convert glucose by aerobic fermentation into partially oxidized end products, mainly succinate and acetate (for review, see Refs. 15 and 16) (Fig. 1A). Most of the glycolysis takes place in specialized peroxisomes, called glycosomes (steps 1–6) (17). In the course of glycolysis, P-enolpyruvate is produced in the cytosol (steps 10–12), where it is located at a branching point. It can re-enter the glycosomes to be converted to succinate within this compartment (steps 13–16) or in the mitochondrion (not shown in Fig. 1) (18, 19). P-enolpyruvate can also be converted into pyruvate (steps 17–18), which enters the mitochondrion to produce acetate (steps 19–22) (20, 21). Acetate is also produced in the mitochondrion from threonine, by the action of four enzymes, i.e. threonine dehydrogenase (TDH) (EC 1.1.1.103, step 23), whose expression is under metabolic control, 2,2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29, step 24), acetyl-CoA thioesterase (ACH, EC 3.1.2.1, steps 22) (11). The two later steps are shared with glucose metabolism (11).

No exchanges of nucleotides or cofactors have been described so far between the glycosomal and cytosolic compartments in T. brucei. Consequently, consumption and production of ATP and NAD⁺ by glycolysis are tightly balanced within the organelle. In the wild-type procyclic trypanosomes, NADH resulting from the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13, steps 6) is re-oxidized inside the organelle by the glycosomal succinic fermentation pathway (steps 14 and 16) (18). In the absence of the succinate branch, such as in the P-enolpyruvate carboxykinase (PEPCK: EC 4.1.1.49, step 13) null mutant (Δpepck), NADH is re-oxidized by glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) with the contribution of the glycerol 3-phosphate/dihydroxyacetone phosphate shuttle and the mitochondrial respiratory chain (steps 7 and 25, in Fig. 1B) (22).

ATP molecules consumed in the upper part of glycolysis (steps 1 and 3) also need to be regenerated inside the glycosomes by the lower part of the pathway. The T. brucei genome encodes five glycosomal kinases directly linked to glycolysis, which could theoretically produce ATP from conversion of triose phosphates inside the organelle (see Fig. 1) (16, 23), i.e. phosphoglycerate kinase isoform C (PGKC: Tb927.1.700) (EC 2.7.2.3, step 9 in Fig. 1D), and isofrom A (PGKA: Tb927.1.720), PEPCK (Tb927.2.4210, step 13), glycerol kinase (Gk: Tb927.9.12550) (EC 2.7.1.30, step 8 in Fig. 1B), and PPDK functioning in the glycolytic direction (Tb927.11.6280, step 17). PGKC is only expressed in bloodstream forms of T. brucei, PGKA shows a low activity in both forms (24, 25) and wild-type procyclic cells do not excrete glycerol from glucose metabolism, implying that PGK isoforms and GK do not contribute significantly to glycosomal ATP production (18, 22). Consequently, PEPCK and PPDK are the only known possible enzymes playing a role in maintaining the glycosomal ATP/ADP balance in the wild-type procyclic cells. The role of PEPCK in this process has clearly been established, since the metabolic flux through the succinate branch represents up to 70% of the glycolytic flux in the wild-type parasite (18, 19, 26). However, the impact of PPDK on glycosomal ATP production is debatable, because metabolic fluxes through this step, in one direction or the other, has not been demonstrated so far.

Here we have investigated the maintenance of the ATP/ADP balance in the organelle by generating and analyzing PPDK (Δppdk) and PPDK/PEPCK (Δppdk/Δpepck) null mutants. Our data show for the first time that PPDK is involved in the maintenance of the glycosomal ATP/ADP balance by functioning in the glycolytic direction.

EXPERIMENTAL PROCEDURES

Growth and Maintenance of Trypanosomes—The procyclic form of T. brucei EATRO1125.T7T (TetR-HYG T7RNAPOL-NEO), which constitutively expresses the T7 RNA polymerase (T7RNAPOL) gene and the tetracycline repressor (TetR) gene under control of a T7 RNA polymerase promoter for tetracycline-inducible expression (27), as well as mutant cell lines, were cultured at 27 °C in SDM79 medium containing 10% (v/v) heat-inactivated fetal calf serum and 3.5 mg/ml of hemin (28). The bloodstream forms of T. brucei 427 90-13 were cultured at 37 °C in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.25 mm β-mercaptoethanol, 36 mm NaHCO₃, 1 mm hypoxanthine, 0.16 mm thymidine, 1 mm sodium pyruvate, 0.05 mm bacitracin, and 2 mm l-cysteine (29).

Gene Knock-out—Replacement of the PEPCK gene (Tb927.2.4210, GeneDB) by the blasticidin (BSD) and puromycin (PAC) resistance markers via homologous recombination was described before (Δpepck cell line, TetR-HYG T7RNAPOL-NEO Δpepck::BSD/Δpepck::PAC) (22). Here, the same plasmid constructs were used to delete the PEPCK alleles in the Δppdk::TetR-HYG/Δppdk::T7RNAPOL-NEO (Δppdk) mutant cell line, in which both PPDK alleles have been replaced by TetR-HYG and T7RNAPOL-NEO genes, respectively (10, 30). The resulting cell line, Δppdk::TetR-HYG/Δppdk::T7RNAPOL-NEO Δpepck::BSD/Δpepck::PAC (Δppdk/Δpepck), was generated by transfection and selection of drug-resistant clones as previously reported (31). The first and second PPDK alleles were replaced by BSD- and PAC-resistant genes, respectively. Transfected cells were selected in SDM79 medium containing hygromycin B (25 μg/ml), neomycin (10 μg/ml), blasticidin (10 μg/ml), and puromycin (1 μg/ml).

Inhibition of PGKB Gene Expression by RNAi—Inhibition of gene expression by RNAi in procyclic trypanosomes (32) was performed by expression of stem-loop “sense/antisense” RNA molecules of the targeted sequences introduced in the pH1336 expression vector (kindly provided by C. Clayton) as previously described (27). To specifically down-regulate expression of the gene encoding the cytosolic phosphoglycerate kinase isoform (PGKB: Tb927.1.710), the pH-PGKB-SAS plasmid was constructed to target a 352-bp fragment corresponding to the PGKB and PGKC (Tb927.1.700) intergenic regions. Briefly, a PCR-amplified 445-bp fragment, containing the antisense PGKB-PGKC intergenic sequence, the first 60 bp of the PGKC gene, and the appropriate restriction sites added to the primers was inserted into HindIII and BamHI restriction sites of the pLew100 plasmid. Then a PCR-amplified fragment containing the sense PGKB-PGKC intergenic sequence (372 bp) was inserted upstream of the antisense sequence, using

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HindIII and Xhol restriction sites (Xhol was introduced at the 3′-extremity of the antisense PCR fragment). Finally, the sense-antisense HindIII/BamHI cassette was inserted in the HindIII/BamHI-digested pHD1336 vector. The resulting plasmid (pHD-PGBK-SAS) contains a sense and antisense version of the targeted gene fragment, separated by a 60-bp fragment (PGKC coding sequence), under control of the procyelic acidic repetitive protein (PARP) promoter linked to a prokaryotic tetracycline (Tet) operator. The RNAiPGKB cell line (TetR-HYG T7RNASOL-NEO RNAiPGKB-BSD) was produced by introducing the pHD-PGBK-SAS plasmid in the EATRO1125.T7T parental cell line. Transfected cells were selected in SM7 medium containing hygromycin B (25 μg/ml), neomycin (10 μg/ml), and blasticidin (10 μg/ml).

Expression of the PGKC and PEPCK Genes—The pLew100 vector (kindly provided by E. Wirtz and G. Cross) (33) was used to express full-length PGKC and PEPCK glycosomal proteins in procyclic trypanosomes by inserting a 1323- or 1578-bp PCR fragment, respectively, in the HindIII and BamHI restriction sites of the plasmid. The resulting pLew-PEPK+ plasmid was inserted in the Δppdk/Δpepck mutant to produce the Δppdk/Δpepck/PEPK+ cell line (Δppdk::TetR-HYG/Δpepck::T7RNASOL-NEO Δpepck::BSD/Δpepck::PAC PEPCK-BLE) and the pLew-PGKC+ plasmid was introduced in the EATRO1125.T7T, Δppdk/Δpepck, and RNAiPGKB cells to generate the PGKC+ (TetR-HYG T7RNASOL-NEO PGKC-BLE), Δppdk/Δpepck/PGKC+ (Δppdk::TetR-HYG/Δpepck::T7RNASOL-NEO Δpepck::BSD/Δpepck::PAC PGKC-BLE), and RNAiPGKB/PGKC+ (TetR-HYG T7RNASOL-NEO RNAiPGKB-BSD PGKC-BLE) cell lines. Transfected cells were selected in SM7 medium containing hygromycin B (25 μg/ml) and neomycin (10 μg/ml), in addition to 2.5 μg/ml of phenomycin (Δppdk/Δpepck/PGKC+ and RNAiPGKB/PGKC+) and/or 1 μg/ml of puromycin (Δppdk/Δpepck/PGKC+).

Enzyme Assays—Threonine 3-dehydrogenase (EC 1.1.1.103) enzyme activity was adapted from Linstead et al. (34). Briefly, cells were washed in PBS, resuspended in hypotonic lysis buffer (5 mM NaHPO4, 0.3 mM KH2PO4), and sonicated (5 s at 4°C). Enzyme assay contained 0.1 mM NADH and ATP/ADP balances, as well as steps involved in ATP production in the respiratory chain. Enzymes of glucose of threonine degradation are indicated in the circled step numbers represent enzymes analyzed here. Excreted end products from degradation of glucose, proline and threonine are boxed (sucinate and alanine are the main end products excreted from proline metabolism, in the presence or absence of glucose, respectively (14)). The arrow thickness is representative of the measured or estimated metabolic flux through the corresponding branches. The rate of glucose and proline consumption (nmol/h/mg of protein) is indicated above and below the carbon source name, respectively (values from Fig. 3). Abbreviations: AcCoA, acetyl-CoA; OA, oxaloacetate; e, electrons; G3P, glyceraldehyde 3-phosphate; Gly3P, glyceraldehyde 3-phosphate; OAA, oxaloacetate; P3G, 3-phosphoglycerate; PE, phosphoenolpyruvate; Pyr, pyruvate; RC, respiratory chain. Enzymes of glucose of threonine degradation are indicated in panel A, or when mentioned below, in panels B or D: 1, hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triose-phosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (panel B); 8, glyceraldehyde-3-phosphate dehydrogenase (panel B); 9, cytosolic phosphoglycerate kinase (PGK); 10, glyceraldehyde-3-phosphate dehydrogenase (panel B); 11, phosphoglycerate mutase; 12, enolase; 13, PEPCK; 14, malate dehydrogenase; 15, cytosolic fumarase (located in the glycosomes for simplification); 16, glyceraldehyde-NADH-dependent fumarate reductase; 17, pyruvate dehydrogenase complex; 20, acelettinate-CoA-transf erase; 21, succinyl-CoA synthetase; 22, acetyl-CoA thioesterase; 23, threonine dehydrogenase (TDH); 24, 2,2-dimethyl-2-hexanoyl-CoA dehydratase; 25, FAD-dependent glyceraldehyde-3-phosphate dehydrogenase (panel B); 26, F2,F,-ATP synthase.

Western Blot Analyses—Total protein extracts of wild-type or mutant procyclic or bloodstream forms of T. brucei (5 × 106 cells) were size-fractionated by SDS-PAGE (10%) or isoelectric focusing gel electrophoresis (Bio-Rad) and immunoblotted on Immobilon-P filters (Millipore) (37). Immunodetection was performed as described (37, 38) using as primary antibodies rabbit anti-PGK (diluted 1:500; gift from P. Michels, Edinburgh, UK, and M. Parsons, Seattle, WA) (39, 40), rat anti-PEPCK (diluted 1:1000; gift from T. Seebeck, Bern, Switzerland) (22), rabbit anti-PPDK (diluted 1:500) (5), rabbit anti-TDH (diluted 1:500) (11), rabbit anti-GPDH (glycerol-3-phosphate dehydrogenase, EC 1.1.1.8; diluted 1:100) (41), mouse anti-β-actin (diluted 1:10,000) (42), and mouse anti-AMP-dependent acetyl-CoA synthetase (EC 6.2.1.1; diluted 1:100) (43) and as secondary antibodies, anti-mouse, anti-rat, or anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, 1:5,000 dilution). Revelation was performed using the SuperSignal West Pico Chemiluminescent Substrate as described by the manufacturer (Thermo Scientific). Alternatively, for quantitative analyses, revelation was performed using the Luminata TM Crescendo Western HRP Substrate (Millipore). Images were acquired and analyzed with a KODAK Image Station 4000MM and quantitative analyses were performed with the KODAK-MI application.

Digitonin Permeabilization—Trypanosomes were washed two times in cold PBS and resuspended at 6.5 × 106 cells/ml (corresponding to 3.3 mg of protein/ml) in STE buffer (250 mM sucrose, 25 mM Tris, pH 7.4, 1 mM EDTA) supplemented with 150 mM NaCl and the Complete™ Mini EDTA-free protease inhibitor mixture (Roche Applied Bioscience). Cell aliquots (200 μl) were incubated with increasing quantities of digitonin (Sigma) for 4 min at 25°C, before centrifugation at 14,000 × g for 2 min to collect the cellular pellet.

Immunofluorescence Analyses—Log phase cells were fixed with formaldehyde as described before (5). Slides were incubated with rabbit anti-PGK (diluted 1:800) (39) and H112 monoclonal antibodies anti-PPDK (undiluted) (5) followed by Alexa Fluor® 594-conjugated goat anti-mouse secondary antibody (diluted 1:100) and/or Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody (diluted 1:100) (Molecular Probes). Cells were viewed with a Leica DM5500B microscope and images were captured by an ORCA®-R2 camera.

FIGURE 1. Schematic representation of the intermediary metabolism of procyclic wild-type and mutant cell lines. This figure highlights steps from the glucose metabolism involved in the maintenance of the glycosomal NAD+/NADH and ATP/ADP balances, as well as steps involved in ATP production in the cytosol and mitochondrion from the three main carbon sources used by the procyclic trypanosomes (glucose, proline, and threonine). For simplification, *indicates when mentioned below, in panels B or D; 1, hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triose-phosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (panel B); 8, glyceraldehyde-3-phosphate dehydrogenase (panel B); 9, cytosolic phosphoglycerate kinase (PGK) (panel D); 10, cytosolic phosphoglycerate kinase (PGK); 11, phosphoglycerate mutase; 12, enolase; 13, PEPCK; 14, malate dehydrogenase; 15, cytosolic fumarase (located in the glycosomes for simplification); 16, glyceraldehyde-NADH-dependent fumarate reductase; 17, pyruvate dehydrogenase complex; 20, acelettinate-CoA-transf erase; 21, succinyl-CoA synthetase; 22, acetyl-CoA thioesterase; 23, threonine dehydrogenase (TDH); 24, 2,2-dimethyl-2-hexanoyl-CoA dehydratase; 25, FAD-dependent glyceraldehyde-3-phosphate dehydrogenase (panel B); 26, F2,F,-ATP synthase.
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(PerkinElmer Life Sciences) (45). Acquisition conditions were as follows: 90° flip angle, 5,000 Hz spectral width, 32 K memory size, and 9.3-s total recycle time. Measurements were performed with 256 scans for a total time close to 40 min. Protons linked to acetaldehyde C2 generate by 1H NMR five resonances, one single peak (13C]acetate) flanked by two doublets (13C]acetate). In both NMR analyses, the intensity of the cells during the incubation was checked by microscopic observation. Measurements were recorded at 25 °C with an ERETIC method, which provides an electronically synthesized reference signal (47). Before each experiment, phase of ERETIC peak was precisely adjusted and, after acquisition, resonances of obtained spectra were integrated and results were expressed relative to ERETIC peak integration.

RESULTS

PPDK Gene Knock-out Affects Acetate Production from Glucose Metabolism—The PPDK null mutant (Δppdk) was previously generated in the wild-type procyclic trypanosomes by replacing both PPDK alleles by neomycin (NEO) and hygromycin (HYG) markers, together with the T7 RNA polymerase (T7RNAPOL) and the tetracycline repressor (TetR) under control of the T7RNAPOL promoter, respectively (10). Western blot analyses confirmed the absence of PPDK expression in the Δppdk mutant (Fig. 2A). Doubling times of Δppdk and wild-type cells are identical (Fig. 2B), however, glucose and proline metabolism of the mutant grown in SDM79 medium is affected (Fig. 3). Indeed the rate of glucose consumption is reduced by 22% in the Δppdk cell line, which is compensated by a 35% increase of the rate of proline consumption compared with wild-type cells (Student’s t test values <0.05). Induction of proline metabolism caused by reduction or abolition of the glycolytic flux was previously described in procyclic trypanosomes (13, 14, 22). Quantitative analyses of 13C-enriched end products excreted from [1,13C]glucose metabolism were performed by 13C NMR. When the wild-type parasite was incubated in PBS/NaHCO3 containing 4 mM [1,13C]glucose, a total of 743 nmol of 13C-labeled metabolites/h/mg of protein were recovered in the supernatant, with most of the label being detected in succinate (63.3%) and acetate (25%), and small amounts of malate, lactate, fumarate, and alanine are also detected (Table 1). We observed that the rate of acetate production decreased in the Δppdk mutant (19.9% of the excreted end products), whereas this value was increased for succinate (70.6% of the excreted end products) (Table 1).

To confirm the role of PPDK in acetate production, we compared threonine and glucose contributions to acetate production in wild-type and Δppdk cell lines. Indeed, a recently developed metabolite profiling assay showed that threonine is the main acetate source of procyclic trypanosomes (11, 46). This approach is based on the ability of 1H NMR spectrometry to distinguish 13C-enriched from 12C molecules, such as [13C]acetate and [12C]acetate derived from uniformly 13C-enriched [U-13C]glucose (4 mM) and unenriched threonine (4 mM), respectively. [13C]Acetate molecules derived from [U-13C]glucose (annotated A13 in Fig. 4A) are represented by two doublets, with chemical shifts at around 2.0 and 1.75 ppm, respectively, whereas the central resonance (1.88 ppm) corresponds to threo-
The rate of acetate production from glucose is reduced by 30% in \( \text{H9004} \) compared with wild-type cells (Student’s \( t \) test values \( P < 0.05 \)), whereas acetate production from threonine remains in the same range (Table 2 and Fig. 4A). It is noteworthy that PPDK contribution to acetate production may be underestimated, because pyruvate can also be generated from malate produced in the glycosomes by the action of the cytosolic and mitochondrial malic enzymes (steps not indicated in Fig. 1A) (48). In the absence of PPDK, contribution of this pathway, as well as pyruvate kinase (step 18), to acetate production could increase. Altogether, these data show that PPDK contributes to acetate production from glucose in wild-type cells, implying that the

FIGURE 2. Production of \( \Delta\text{ppdk}, \Delta\text{ppdk}/\Delta\text{pepck}, \) and rescue cell lines. Panel A shows a Western blot analysis of the parental procyclic (WT) and mutant cell lines with the immune sera indicated in the right margin. Panel B shows growth curves of the wild-type (WT), \( \Delta\text{ppdk}, \Delta\text{pepck}, \Delta\text{ppdk}/\Delta\text{pepck}, \) and tetracycline-induced (\( \text{ni} \)) and uninduced (\( \text{ni} \)) \( \Delta\text{ppdk}/\Delta\text{pepck}/\text{PEPCK}\text{.ni} \) and \( \Delta\text{ppdk}/\Delta\text{pepck}/\text{PEPCK}\text{.ni} \) cell lines, as well as a Western blot analysis of the wild-type (\( \blacksquare \)), \( \Delta\text{ppdk}/\Delta\text{pepck}/\text{PEPCK}\text{.ni} \), and \( \Delta\text{ppdk}/\Delta\text{pepck}/\text{PEPCK}\text{.ni} \) cell lines with the immune sera indicated in the right margin of the inset. Panel C shows a PCR analysis of genomic DNA isolated from the parental wild-type and \( \Delta\text{ppdk}, \Delta\text{pepck}, \) and \( \Delta\text{ppdk}/\Delta\text{pepck} \) cell lines. Amplifications were performed with primers based on sequences that flank the 5′ UTR and 3′ UTR fragments used to target the PEPCK gene depletion (\( \blacksquare \)) and internal sequences of the blasticidin (BSD, PCR products 3 and 4), puromycin (PAC, PCR products 5 and 6) resistance genes and, as controls, the PEPCK gene (products 1 and 2). As expected, PCR amplification of the PEPCK gene was only observed in wild-type and \( \Delta\text{ppdk} \) cell lines, whereas BSD and PAC PCR products were observed only in \( \Delta\text{pepck} \) and \( \Delta\text{ppdk}/\Delta\text{pepck} \) cell lines. White stars indicate the expected PCR fragment.
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TABLE 1
Excreted end products of glucose metabolism by procycl T. brucei cell lines

| Cell linea | n  | Succinate | Acetate | Lactate | Malate | Fumarate | Alanine | β-hydroxybutyrate | Pyruvate | Glycerol | Total |
|------------|----|-----------|---------|---------|--------|----------|---------|-------------------|----------|----------|-------|
| WT (EATRO1125.T7T) | 7  | 5.50 ± 15 | 21.82 ± 15 | 20.00 ± 17 | 18.00 ± 17 | ND | 0.70 ± 1.7 | 21.00 ± 21 | 18.00 ± 18 | 20.00 ± 20 | 18.00 ± 18 |
| Δppdk | 3  | 0.60 ± 11 | 14.10 ± 11 | 13.70 ± 6.4 | 22.20 ± 1.1 | 6.20 ± 3.9 | ND | 18.00 ± 18 | 18.00 ± 18 | 20.00 ± 20 | 18.00 ± 18 |
| Δpepcke | 3  | 1.20 ± 14 | 15.40 ± 14 | 18.00 ± 3.7 | ND | 14.00 ± 8.7 | 8.50 ± 4.4 | ND | 18.00 ± 18 | 18.00 ± 18 | 20.00 ± 20 | 18.00 ± 18 |
| Δppdk/Δpepcke | 3  | ND | 7.00 ± 10 | ND | 0.70 ± 2.1 | 21.00 ± 21 | 18.00 ± 18 | 20.00 ± 20 | 18.00 ± 18 | 20.00 ± 20 | 18.00 ± 18 |
| PGKC+/ni (1 day) | 1  | 535.0 ± 4 | 151.0 ± 11 | 30.5 ± 5.5 | 5.0 ± 2.3 | 1.0 ± 1.4 | ND | 0.8 ± 0.7 | 0.5 ± 0.5 | 1.2 ± 1.2 | 2.0 ± 2.0 |
| PGKC+/ni (2 days) | 3  | 91.0 ± 18 | 118.0 ± 33 | 35.8 ± 9.2 | 4.2 ± 3.3 | ND | 3.4 ± 1.2 | 22.0 ± 22 | 1.7 ± 2.0 | 3.1 ± 3.1 | 0.7 ± 0.7 |
| Δppdk/Δpepck/PGKC+ | 3  | 2.90 ± 0.5 | 181.0 ± 14 | 108.0 ± 9.6 | ND | 7.8 ± 1.1 | 9.2 ± 1.6 | ND | 16.4 ± 2.0 | 325 ± 325 | 28 ± 28 |

a: ± RNAi cell lines tetracycline-induced during 1 to 3 days depending on the cell line and the experiments; ni: non-induced RNAi cell lines.

Glucose Metabolism Is Strongly Impaired in the Δppdk/Δpepcke Mutant—According to the current model, PEPCK and PPDK are the main glycosomal kinase candidates involved in the maintenance of the glycosomal ATP/ADP balance, as proposed by deletion of the PEPC gene in the PPDK null background (Δppdk). Both PEPC alleles were replaced by puromycin (PAC) and blasticidin (BSD) markers to generate the Δppdk/Δpepcke cell line. Deletion of both PEPC alleles in the PPDK null background was confirmed by Western blot (Fig. 2A) and PCR analyses (Fig. 2C). The resulting Δppdk/Δpepcke cell line is viable although it shows an increased doubling time (14.5 h) compared with the wild-type (11.9 h), Δppdk (12 h), and Δpepcke (11.8 h) cell lines (Fig. 2B). A conditional re-expression of an ectopic copy of the PEPC gene in the PPDK null background was performed to produce the Δppdk/Δpepcke/PEPC+ cell line. Addition of tetracycline restored the wild-type growth phenotype of the Δppdk/Δpepcke/PEPC+ cell line (Fig. 2B). Upon re-expression of PEPC as shown by Western blot analysis (Fig. 2B, inset).

To determine the effect of PPDK and/or PEPC gene depletion on the glycosomal metabolism, the rate of glucose and proline consumption was measured for all these cell lines grown in SDM79 medium (Fig. 3). As previously observed, the Δpepcke mutant showed a strong reduction of glucose consumption (70%) compensated by a 90% increase in proline consumption (22). The Δppdk/Δpepcke mutant still consumes glucose, although with a moderately reduced rate compared with the Δpepcke mutant (1.5-fold), whereas the rate of proline consumption is not affected. The absence of compensation toward proline metabolism may explain the reduced growth rate of the Δppdk/Δpepcke cell line. As expected, re-expression of PEPC in the Δppdk/Δpepcke cell line (Δppdk/Δpepcke/PEPC+) restored the rate of glucose and proline consumption observed in the Δppdk cell line (Fig. 3). To confirm these data, a 13C-NMR quantitative analysis of 13C-enriched end products excreted from [1-13C]glucose metabolism was performed. Similar results have been obtained for the Δppdk and Δppdk/Δpepcke/PEPC+ cell lines (Table 1). In contrast, succinate production is abolished in the Δpepcke mutant, whereas acetate production is not affected, with a 3.6-fold reduction of end product excretion from the glucose metabolism (Table 1) (22). Interestingly, the Δppdk/Δpepcke mutant excreted 2.8-fold less end products from glucose metabolism compared with the Δpepcke mutant (Table 1), suggesting that glycolysis of the double mutant is strongly impaired when glucose is the only carbon source. In other words, the PPDK activity appears critical to maintain glycolytic activity in the PEPC null background.

Comparison of Glucose and Threonine Metabolism in the Δpepcke and Δppdk/Δpepcke Mutants—To determine why glycolysis is more affected in the Δppdk/Δpepcke double mutant than in the Δpepcke single mutant, we compared acetate production from glucose and threonine in both cell lines. We previously observed that deletion of the PEPC gene induced a down-regulation of expression of the TDH gene and activity, leading to a reduction of threonine contribution to acetate production (Fig. 4) (11). We have interpreted this TDH down-regulation as a consequence of an increased metabolic flux from glucose metabolism toward the acetate branch, due to abolition of the succinate branch in the Δpepcke mutant (see Fig. 1B). In addition, the Δpepcke mutant, but not the wild-type cells, excretes detectable amounts of β-hydroxybutyrate from glucose metabolism, which was probably derived from accumulation of acetyl-CoA (22) (Fig. 5 and Table 1). Altogether, these data suggest that redirection of the glycolytic flux toward acetate production induces accumulation of acetyl-CoA, which directly or indirectly induces down-regulation of TDH expression to reduce contribution of threonine degradation to acetyl-CoA production (see Fig. 1B). This is probably due to the limited capacity of acetate production, as previously proposed (50). The same analyses revealed that the Δppdk/Δpepcke cell line behaves differently. First, analysis of [1-13C]glucose metabolism, as the only carbon source, showed no evidence of β-hy-
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Excreted acetate from glucose and threonine metabolism by procyclic T. brucei cell lines

The spent PBS medium of trypanosome cell lines incubated in the presence of 4 mM threonine and 4 mM [U-13C]glucose was analyzed by 1H NMR spectrometry to detect and quantify excreted acetate.

| Cell lines | Acetate production from threonine | Acetate production from glucose | Student’s t test (compared with WT)* |
|------------|----------------------------------|---------------------------------|-------------------------------------|
| WT         | 38 ± 561                         | 1240 ± 250                      | /                                   |
| Δpepck     | 11 ± 215                         | 1030 ± 136                      | 0.01042                             |
| Δppdk      | 3 ± 316                          | 863 ± 39                        | 0.01370                             |
| Δppdk/Δpepck | 6 ± 295                       | 836 ± 123                       | 0.00038                             |

A number of biological replicates.

* Statistical significance for the difference in the acetate production levels from glucose between the EATRO1125.T7T (WT) cells and the other cell lines was determined using the Student’s t test (bilateral and equal variance parameters). Statistical differences are significant for values <0.05.

FIGURE 4. TDH expression and activity are not reduced in the Δppdk/Δpepck cell line. In panel A, contribution of threonine and glucose to acetate production was determined by 1H NMR analysis. Acetate excreted by the procyclic wild-type (WT), Δppdk, Δpepck, and Δppdk/Δpepck cell lines from 4 mM 6-[U-13C]glucose and 4 mM threonine was determined by 1H NMR. Each spectrum corresponds to one representative experiment from a set of at least 3. A part of each spectrum ranging from 1.6 to 2.1 ppm is shown. The resonances were assigned as indicated: A12, threonine-derived acetate; A13, 13C-enriched glucose-derived acetate. Panel B shows the TDH activity (milliunits/mg of protein), normalized with the pyruvate dehydrogenase activity measured in the same samples. In panel C, expression of TDH and glycerol-3-phosphate dehydrogenase (GPDH) was analyzed by Western blotting with specific immune sera. The ratio between the TDH and GPDH signals, indicated below the blot, represents a mean ± S.D. of 4 different experimental duplicates, with an arbitrary value of 1 for the parental cells (WT).

FIGURE 5. Analysis of β-hydroxybutyrate and glycerol production from glucose. Supernatant of wild-type (WT) and mutant cell lines incubated with 4 mM [1-13C]glucose were analyzed by 13C NMR. The resonances were assigned as follows: A, acetate; B, β-hydroxybutyrate; G, glycerol; G6, carbon C6 of glucose; L, lactate; M, malate; AG, acylglycerol; S, succinate.

regulation of TDH (11). Interestingly, high threonine contribution to acetate production is restored in Δppdk/Δpepck cells, which correlates with the wild-type expression level of TDH (Fig. 4A and Table 2).

Altogether these data suggest that the reasons for glycolytic down-regulation differ in the Δpepck and Δppdk/Δpepck cell lines. As mentioned above, deletion of the PEPCK gene induces a redistribution of the metabolic flux toward the acetate branch, with as a consequence accumulation of intermediary metabolites (such as β-hydroxybutyrate), reduction of the glycolytic flux, and down-regulation of the TDH activity. In the Δppdk/Δpepck mutant, TDH activity remains high and production of β-hydroxybutyrate from glucose is not detectable, suggesting that the acetate branch is not overloaded. Consequently, the reason for glycolysis down-regulation may reside inside the glycosomes, such as the imbalanced ATP/ADP ratio. This hypothesis is strengthened by the important increase of glycerol production in the Δppdk/Δpepck mutant. Indeed, 13C NMR
analysis of the [1-13C]glucose metabolism showed that [13C]glycerol represents 23 and 3% of the 13C-enriched end products excreted from [1-13C]glucose in the Δppdk/Δpepck and Δpepck mutants, respectively, whereas it is not detectable in wild-type cells (Table 1 and Fig. 5). Increase of glycerol production in the Δppdk/Δpepck mutant by the ATP generating glycolysis is certainly induced to compensate for the simultaneous loss of PPDK and PEPC kinase activities (see Fig. 1C), which strongly supports the role of PPDK in intraglycosomal ATP production.

**Expression of the Glycosomal rPGKC Is Lethal for the Procyclic Trypanosomes**—To restore glycosomal ATP production, the recombinant glycosomal phosphoglycerate kinase (rPGKC), which has never been detected in procyclic cells, was conditionally overexpressed in the procyclic forms of *T. brucei* (rPGKC), which has never been detected in procyclic cells, was conditionally overexpressed in the procyclic cells (Fig. 1—To restore glycosomal ATP production. The growth curves of the parental (WT) and tetracycline-induced (.i) mutant procyclic cell lines expressing rPGKC in the wild-type (WT) and tetracycline-induced (i) mutant procyclic cell lines expressing rPGKC in the wild-type, PGKC+.ni, and PGKC+.i, respectively. Because PGKB, the PGK isoform expressed in the cytosol of procyclic trypanosomes, and PGKC have the same apparent electrophoretic mobility in SDS-PAGE, but different PK, (PGKC, 9.25; PGKB, 7.11), both proteins have been separated by isoelectric focusing gel electrophoresis. As expected, the bloodstream and procyclic forms was analyzed by Western blot on total cellular protein extracts separated by SDS-PAGE (Fig. 6C). The PGKC+.i cell line expresses similar amounts of both PGK isoforms, whereas the recombinant PGK isoform (rPGKC) is not detectable in the PGKC+.ni cells (Fig. 6C).

Because of the presence of high amounts of PGKB in the cytosol of the procyclic cells, we determined the cellular localization of rPGKC in procyclic cells down-regulated for expression of PGKB by RNAi (PGKB/PGKC+.i cell line). Down-regulation of PGKB to a very low level of expression (Fig. 7B) does not affect growth of the PGKB/i parasites (Fig. 7A), whereas the PGKB/PGKC+.i mutant declined 1 day post-induction and died within the next 2 weeks (Fig. 7). Immunofluorescence analyses of the PGKB/PGKC+.i cell line with the anti-PGK antibodies showed a punctuate glycosomal-like pattern overlapping with the anti-PPDK glycosomal marker, whereas wild-type cells showed a diffuse cytosolic-like pattern (Fig. 7C). The glycosomal localization of rPGKC was confirmed by a cellular fractionation experiment wherein the different membranes of the procyclic trypanosomes were differentially permeabilized by increasing concentrations of the detergent digitonin. A Western blot analysis of the pellet fractions indicates that the PGK isoform detected in the wild-type parasites (PGKB) is released together with the cytosolic marker, acetyl-CoA synthetase (43), at 40 μg of digitonin/mg of protein, whereas the PPDK glycosomal marker is released at much higher digitonin concentrations (300 μg of digitonin/mg of protein) (Fig. 7D, upper panel). However, the PGK isoform expressed in the PGKB/PGKC+.i cell line is released together with PPDK, confirming that rPGKC is expressed in the glycosomes (Fig. 7D, lower panel).

**Expression of rPGKC Affects Succinate and ATP Production**—To determine why expression of rPGKC is lethal for the procyclic forms, we down-regulated the expression of PGKB by RNAi (PGKB/PGKC+.i cell line). Down-regulation of PGKB to a very low level of expression (Fig. 7B) does not affect growth of the PGKB/i parasites (Fig. 7A), whereas the PGKB/PGKC+.i mutant declined 1 day post-induction and died within the next 2 weeks (Fig. 7). Immunofluorescence analyses of the PGKB/PGKC+.i cell line with the anti-PGK antibodies showed a punctuate glycosomal-like pattern overlapping with the anti-PPDK glycosomal marker, whereas wild-type cells showed a diffuse cytosolic-like pattern (Fig. 7C). The glycosomal localization of rPGKC was confirmed by a cellular fractionation experiment wherein the different membranes of the procyclic trypanosomes were differentially permeabilized by increasing concentrations of the detergent digitonin. A Western blot analysis of the pellet fractions indicates that the PGK isoform detected in the wild-type parasites (PGKB) is released together with the cytosolic marker, acetyl-CoA synthetase (43), at 40 μg of digitonin/mg of protein, whereas the PPDK glycosomal marker is released at much higher digitonin concentrations (300 μg of digitonin/mg of protein) (Fig. 7D, upper panel). However, the PGK isoform expressed in the PGKB/PGKC+.i cell line is released together with PPDK, confirming that rPGKC is expressed in the glycosomes (Fig. 7D, lower panel).
The fate of the carbon sources metabolism was analyzed 1 and 2 days post-induction, when the cells were still highly mobile. After 3 days post-induction, cell motility and viability were affected, with possible secondary effects on the intermediate and energy metabolism. The rate of glucose and proline consumption was measured for the PGKC\(^+/\)H11001 cell line grown in the SDM79 medium (Fig. 3). Two days post-induction, the rate of glucose consumption is reduced by 42% with no increase of the proline metabolism to compensate for the reduced glycolysis. The reduced glycolytic flux was confirmed by the 25 and 53% reduction of glucose-derived excreted end products after 1 and 2 days of induction, respectively, compared with uninduced cells (Table 1). Interestingly, the rate of acetate production is only moderately affected (7 and 22% of reduction) compared with succinate (41 and 74% of reduction) after 1 and 2 days of induction, respectively (Table 1 and Fig. 8). The 4-fold reduction of succinate production, although acetate production is poorly affected, is probably related to the maintenance of the glycosomal ATP/ADP balance. Indeed, one may expect that, to prevent accumulation of ATP in the organelle, reduction of ATP production by PEPCK and PPDK should compensate for glycosomal ATP production by rPGKC, with as a consequence a reduced metabolic flux in the succinate branch (see Fig. 1D). Another consequence of rPGKC expression in the procyclic trypanosomes, the fate of the carbon sources metabolism was analyzed 1 and 2 days post-induction, when the cells were still highly mobile. After 3 days post-induction, cell motility and viability were affected, with possible secondary effects on the intermediate and energy metabolism. The rate of glucose and proline consumption was measured for the PGKC\(^+/\)H11001 cell line grown in the SDM79 medium (Fig. 3). 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Indeed, one may expect that, to prevent accumulation of ATP in the organelle, reduction of ATP production by PEPCK and PPDK should compensate for glycosomal ATP production by rPGKC, with as a consequence a reduced metabolic flux in the succinate branch (see Fig. 1D). Another consequence of rPGKC expression in the
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The reduced rate of proline consumption is probably the consequence of the increased glycolytic flux, as we previously observed for wild-type cells (13, 14). However, as observed for the RNAi PGKC.i cell line, the intracellular ATP concentration was ~2-fold reduced after 2 days of rPGKc induction compared with wild-type cells (Table 3). This was probably due, as proposed above for the RNAi PGKC.i mutant, to the glycosomal redistribution of PKG-dependent ATP production leading to cellular ATP depletion and cell death (see Fig. 1E). It is noteworthy that the leaky expression of rPGK in the Δppdk/Δpepck RNAi PGKC.ni cell line (data not shown) is probably responsible for the reduced intracellular ATP concentration (Table 3) and the reduced growth rate (Fig. 6A) of the uninhibited cells.

**DISCUSSION**

According to the current model, the glycosomal ATP/ADP balance has to be maintained by glycosomal kinase activities to regenerate ATP molecules consumed in the upper part of glycolysis. These kinase activities are essential, because compartmentalization of glycolysis inside the glycosomes is required to prevent a lethal turbo-explosion of glycolysis in trypanosomes (51, 52). This function is ensured by PGK in the bloodstream trypanosomes (53). In the procyclic trypanosomes, the succinate branch plays this role with ATP being produced by PEPCK, whereas the role of PPDK in ATP production was proposed but not experimentally addressed (6, 7). Here we show that in the absence of PEPCK, PPDK participates in the maintenance of glycolytic flux by providing ATP. The evidence comes from the reduction of glucose-derived acetate production in the Δppdk mutant, which can only be interpreted as a consequence of PPDK functioning in the glycolytic direction with production of ATP inside the glycosomes (see Fig. 1A). Comparison of Δppdk/Δpepck and Δpepck metabolism and restoration of glycosomal ATP production by expression of the glycosomal PGK isoform in the Δppdk/Δpepck background further supports the role of PPDK in maintaining the ATP/ADP balance. We previously demonstrated that in the absence of PEPCK the reduced glycolytic flux results from abolition of the succinate branch associated with the limited capacity of the acetate branch (11, 50) (see Fig. 1B). When glucose is the only carbon source available, glycolytic flux is strongly affected in the Δppdk/Δpepck cell line, with a ~3-fold reduction of acetate production, whereas glycerol production is ~3-fold increased, compared with the Δpepck cell line. Consequently, the rates of glycerol and acetate production are in the same range in the double mutant (40 versus 17 nmol/h/mg of protein) (Table 1). These data are in agreement with (i) glycerol kinase activity substituting PPKD activity to produce ATP, in the PEPCK null background and (ii) PPKD being a more successful step, versus glycerol kinase, to balance the ATP/ADP ratio in the procyclic trypanosomes. The role of PPKD in glycosomal ATP production was confirmed by a functional rescue. Indeed, expression of the glycosomal PGK isoform restored the glycolytic rate, with a 4.4-fold increase compared with the Δppdk/Δpepck cell line (Table 1).

Interestingly, PPKD is required to maintain a high glycolytic flux, as shown by the 22 and 24% reduction of the rate of glucose

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**TABLE 3**

Intracellular ATP concentration

ATP concentrations (nmol/mg of protein) were determined on trypanosome extracts using the firefly luciferase bioluminescence assay.

| Cell lines      | Nmol of ATP/mg of protein | Student’s t test (compared with WT) |
|-----------------|---------------------------|-----------------------------------|
| WT              | 4.10 ± 0.41               |                                    |
| Δppdk           | 3.53 ± 0.38               | 0.00009                           |
| Δpepck          | 3.98 ± 0.55               | 0.54389                           |
| Δppdk/Δpepck    | 3.55 ± 0.91               | 0.03776                           |
| PGKC.ni         | 8.37 ± 1.32               | 0.33238                           |
| PGKC.i (1 day)  | 5.25 ± 0.69               | 0.00001                           |
| PGKC.i (2 days) | 1.84 ± 0.30               | 5.65 10^-11                       |
| Δppdk/Δpepck/PGKC.ni | 2.09 ± 0.56 | 2.53 10^-9                       |
| Δppdk/Δpepck/PGKC.i (1 day) | 1.72 ± 0.24 | 2.29 10^-13                       |
| Δppdk/Δpepck/PGKC.i (2 days) | 1.51 ± 0.19 | 1.95 10^-14                       |
| RNAiPGKB.ni     | 8.07 ± 0.71               | 0.89509                           |
| RNAiPGKB.i      | 7.38 ± 0.58               | 0.19717                           |

* Number of biological replicates.
* a Statistical significance for the difference in the ATP levels between the EATRO1125.TT (WT) cells and the other cell lines was determined using the student’s t test (bilateral and equal variance parameters). Statistical differences are significant for values <0.05.
* b Not significant.

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glycosomes of the procyclic cells is the reduced net production of ATP from glycolysis, due to reduction of PGKB contribution to cytosolic ATP production (see Fig. 1D). In agreement with this hypothesis, the intracellular ATP concentration was reduced by 38 and 51% after 1 and 2 days of rPGKc induction, respectively, compared with the wild-type cells (Table 3), although amounts of intracellular ATP remain high in the PGKC.i mutant, which can only be interpreted as a consequence of PPDK functioning in the glycolytic direction with production of ATP inside the glycosomes (see Fig. 1A). Expression of rPGK Restores Glycolysis in the Δppdk/Δpepck Mutant—The same analyses were conducted on the Δppdk/Δpepck RNAi PGKc cell line. Two days post-induction, the rate of glycolytic ATP production is 2.2-fold increased versus 1.9-fold decrease of proline consumption, compared with the uninhibited cells (Fig. 3). The increased glycoytic flux was confirmed by 1H NMR analyses because the rate of 13C-enriched end product excretion from [1-13C]glucose metabolism, when glycolysis is the only carbon source, is 4.5-fold increased compared with the parental Δppdk/Δpepck cell line (Table 1). It is noteworthy that, in these incubation conditions, the rate of acetate production is fully restored in the Δppdk/Δpepck RNAi PGKc.i mutant compared with wild-type cells (181 ± 14 versus 186 ± 21 nmol/h/mg of protein). Because the succinate branch is abolished in the Δppdk/Δpepck RNAi PGKc.i cell line, restoration of the flux through the acetate branch suggests that the maximal glycolytic capacity in the PPDK/PEPCK null background has been restored by rPGKc expression. Consequently, providing additional ATP molecules in the glycosomes, through the rPGKc activity, restores glycolytic flux in the PPDK/PEPCK null background. This further confirms that the dramatic reduction of glycosomal ATP production is the main reason of glycolytic flux down-regulation in the Δppdk/Δpepck mutant.
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consumption in the Δppdk and Δppdk/Δpepck/PEPCK+ i cell lines, respectively. This may be due to intraglycosomal accumulation of PPI, which could affect glycolysis, because no pyrophosphatase activity has been detectable in glycosomal extracts of trypanosomes so far (6). This hypothesis is not supported by the recent discovery of glycosomal pores large enough for diffusion of PPI (49), which can be hydrolyzed by the cytosolic or acidocalcisomal pyrophosphatase activities (54, 55). Alternatively, the role of PPDK in the maintenance of a high glycolytic flux may be related in its PPI dependence. The PPI-dependent glycolytic enzymes, PPDK and phosphofructokinase (PP-FrK), are expressed in a number of parasites to increase the energy efficiency of glycolysis. Indeed, the use of PPI as energy substrate increases the rate of ATP production from glucose 2.5-fold, assuming that PPI is a by-product of the biosynthetic reaction that is commonly wastefully hydrolyzed by pyrophosphatase activity (56). Thus, the trypanosome PPDK can theoretically produce 2 molecules of ATP/molecule of P-enolpyruvate consumed, if adenylate kinase (2 ADP → ATP + AMP) and the absence of a glycosomal pyrophosphatase activity are integrated in the equation. Indeed, in the presence of a glycosomal adenylate kinase activity, which was well described in trypanosomes (57), the PPDK reaction (P-enolpyruvate + AMP + PPI → ATP + PPI + pyruvate) can be written as follows: P-enolpyruvate + 2 ADP + PPI → 2 ATP + PPI + pyruvate. Considering that PPDK produces 2 times more ATP per P-enolpyruvate consumed than PEPCK, higher is the contribution of PPDK compared with PEPCK, lower is the need to metabolize P-enolpyruvate in the glycosomes to maintain the organelar ATP/ADP balance. This implies that the PPDK contribution favors a metabolic flux redistribution toward pyruvate kinase, with an associated increase of cytosolic ATP production (see Fig. 1A). We propose that the main role of PPDK in the procyclic trypanosomes grown in glucose-rich conditions is to increase the rate of intraglycosomal ATP production by taking advantage of the PP, high-energy bond produced by glycosomal biosynthetic pathways (49, 58), to increase the net production of ATP in the cytosol. Thus, the net yield of ATP production per molecule of glucose consumed may depend on the involvement of PPDK versus PEPCK.

Altogether, the reduced glycolytic flux combined with the reduced efficiency of glycolysosomal ATP production, in the absence of the PPDK gene, may induce a reduction of cytosolic ATP production, which is compensated by an increase of proline consumption (36 and 28% in the Δppdk and Δppdk/Δpepck/PEPCK+ i cell lines, respectively). Induction of proline metabolism has previously been observed in trypanosomes, in response to glucose depletion (13, 14) or reduction of the glycolytic flux caused by PEPCK gene deletion (22) or down-regulation of the glycosomal fumarate reductase (18, 19). Indeed, proline can substitute for glucose depletion to feed the central metabolism and produce ATP molecules. Proline is metabolized in the mitochondrion where it produces ATP by substrate-level phosphorylation (succinyl-CoA synthetase) and oxidative phosphorylation as a consequence of respiratory chain-mediated oxidation of proline-derived reducing equivalents (59).

Expression of the rPGKC in the PEPCK/PPDK null and wild-type backgrounds is lethal 2–3 days post-induction because of intracellular ATP depletion (Table 3). The PGK substrate (1,3-biphosphoglycerate) is synthesized in the glycosomes where it can be metabolized by rPGKC before reaching the cytosol where PGKB is located. Thus, we propose that relocation of the PGK activity in the glycosomes leads to ATP deprivation in the cytosol, which is not compensated by an increase of the rate of proline consumption to increase ATP production in the mitochondrion. The absence of a switch toward proline metabolism to increase ATP production is probably related to the remaining high glycolytic flux in PGKC+ i and Δppdk/Δpepck/PGKC+ i cell lines. Indeed, expression of PGKC does not have a direct effect on the wild-type glycolytic flux, because its expression in the PEPCK/PPDK null background stimulates glycolysis. The observed 1.7-fold reduction of glycolytic flux in the PGKC+ i mutant, although the cells start to decline after 2 days of induction, is probably the consequence of ATP deprivation (see Table 3). This strongly supports the view that a high glycolytic flux prevents a switch toward proline metabolism, even if ATP production from glycolysis is impaired. The same phenotype was also reported before for the pyruvate kinase mutant (step 13 in Fig. 1A) (10). This implies that intracellular amounts of ATP are not the driven force leading to metabolic switch to proline metabolism, observed in the absence of glucose or in mutants showing a reduced glycolytic flux.

Manipulating PGK expression is also detrimental for bloodstream forms of T. brucei, because expression of the cytosolic PGKB isoform, whereas the glycosomal PGKC isoform is endogenously expressed, is lethal for the parasite (53). It was proposed that cell death is caused by the reduced glycolytic flux consecutive of imbalance in the glycosomal ADP/ATP ratio. This highlights the central and different role of PGK in the metabolism of these two forms, maintaining the glycososomal ATP/ADP balance in the bloodstream forms and net production of cytosolic ATP in the procyclic cells.

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REFERENCES

1. Marshall, J. S., Ashton, A. R., Govers, F., and Hardham, A. R. (2001) Isolation and characterization of four genes encoding pyruvate, phosphate dikinase in the oomycete plant pathogen Phytophthora cinnamomi. Curr. Genet. 40, 73–81
2. Nevalainen, L., Hrdý, J., and Müller, M. (1996) Sequence of a Giardia lamblia gene coding for the glycolytic enzyme, pyruvate, phosphate dikinase. Mol. Biochem. Parasitol. 77, 217–223
3. Feng, X. M., Cao, L. J., Adam, R. D., Zhang, X. C., and Lu, S. Q. (2008) The catalyzing role of PPDK in Giardia lamblia. Biochem. Biophys. Res. Commun. 367, 394–398
4. Saavedra, E., Encalada, R., Pineda, E., Jasso-Chávez, R., and Moreno-Sánchez, R. (2005) Glycolysis in Entamoeba histolytica. Biochemical characterization of recombinant glycolytic enzymes and flux control analysis. FEBS J. 272, 1767–1783
5. Bringuaud, F., Baltz, D., and Baltz, T. (1998) Functional and molecular characterization of a glycosomal PPI-dependent enzyme in trypanosomatids: pyruvate, phosphate dikinase. Proc. Natl. Acad. Sci. U.S.A. 95,
The mitochondrion is the essential precursor of lipid biosynthesis in procyclic trypanosomes. Proc. Natl. Acad. Sci. U.S.A. 106, 12694–12699

44. Bates, L. S., Waldren, R. P., and Teare, I. D. (1973) Rapid determination of free proline for water-stress studies. Plant and Soil 39, 205–207

45. Lemasters, J. J., and Hackenbrock, C. R. (1979) Continuous measurement of adenosine triphosphate with firefly luciferase luminescence. Methods Enzymol. 56, 530–544

46. Mazet, M., Morand, P., Biran, M., Bouyssou, G., Courtois, P., Daulouède, S., Milleriou, Y., Franconi, J. M., Vincendeau, P., Moreau, P., and Bringaud, F. (2013) Revisiting the central metabolism of the bloodstream forms of Trypanosoma brucei: production of acetate in the mitochondrion is essential for parasite viability. PLoS Negl. Trop. Dis. 7, e2587

47. Akoka, S., Barantin, L., and Trierweiler, M. (1999) Concentration measurement by proton NMR using the ERETIC method. Anal. Chem. 71, 2554–2557

48. Allmann, S., Morand, P., Ebikeme, C., Gales, L., Biran, M., Hubert, J., Brennand, A., Mazet, M., Franconi, J. M., Michels, P. A., Portais, J. C., Boshart, M., and Bringaud, F. (2013) Cytosolic NADPH homeostasis in glucose-starved procyclic Trypanosoma brucei relies on malic enzyme and the pentose phosphate pathway fed by gluconeogenic flux. J. Biol. Chem. 288, 18494–18505

49. Gualdrón-López, M., Brennand, A., Hannaert, V., Quiñones, W., Cáceres, A. J., Bringaud, F., Concepción, J. L., and Michels, P. A. (2012) When, how and why glycolysis became compartmentalised in the Kinetoplastea. A new look at an ancient organelle. Int. J. Parasitol. 42, 1–20

50. Milleriou, Y., Morand, P., Biran, M., Mazet, M., Moreau, P., Wargnies, M., Ebikeme, C., Derramchia, K., Gales, L., Portais, J. C., Boshart, M., Franconi, J. M., and Bringaud, F. (2012) ATP synthesis-coupled and -uncoupled acetate production from acetyl-CoA by the mitochondrial acetate: succinate CoA-transferase and acetyl-CoA thioesterase in Trypanosoma. J. Biol. Chem. 287, 17186–17197

51. Furuya, T., Kessler, P., Jardim, A., Schnaufer, A., Cruder, C., and Parsons, M. (2002) Glucose is toxic to glycosome-deficient trypanosomes. Proc. Natl. Acad. Sci. U.S.A. 99, 14177–14182

52. Haanstra, J. R., van Tuijl, A., Kessler, P., Reijnders, W., Michels, P. A., Westerhoff, H. V., Parsons, M., and Bakker, B. M. (2008) Compartmentation prevents a lethal turbo-explosion of glycolysis in trypanosomes. Proc. Natl. Acad. Sci. U.S.A. 105, 17718–17723

53. Blattner, J., Helfert, S., Michels, P., and Clayton, C. (1998) Compartmentation of phosphoglycerate kinase in Trypanosoma brucei plays a critical role in parasite energy metabolism. Proc. Natl. Acad. Sci. U.S.A. 95, 11596–11600

54. Lemercier, G., Dutoya, S., Luo, S., Ruiz, F. A., Rodrigues, C. O., Baltz, T., Docampo, R., and Bakalara, N. (2002) A vacuolar-type H+-pyrophosphatase governs maintenance of functional acidocalcisomes and growth of the insect and mammalian forms of Trypanosoma brucei. J. Biol. Chem. 277, 37369–37376

55. Lugnibuehl, E., Kunz, S., Wentzinger, L., Freimoser, F., and Seebeck, T. (2011) The exopolyphosphatase TbrPPX1 of Trypanosoma brucei. BMC Microbiol. 11, 4

56. Mertens, E. (1993) ATP versus pyrophosphate: glycolysis revisited in parasitic protists. Parasitol. Today 9, 122–126

57. Ginger, M. L., Ngazoa, E. S., Pereira, C. A., Pullen, T. J., Kabiri, M., Becker, K., Gull, K., and Steverding, D. (2005) Intracellular positioning of isoforms explains an unusually large adenylate kinase gene family in the parasite Trypanosoma brucei. J. Biol. Chem. 280, 11781–11789

58. Michels, P. A., Hannaert, V., and Bringaud, F. (2000) Metabolic aspects of glycosomes in Trypanosomatidae: new data and views. Parasitol. Today 16, 482–489

59. Bringaud, F., Barrett, M. P., and Zilberstein, D. (2012) Multiple roles of proline transport and metabolism in trypanosomatids. Front. Biosci. 17, 349–374