ATP Generation in the Trypanosoma brucei Procylic Form

CYTOSOLIC SUBSTRATE LEVEL PHOSPHORYLATION IS ESSENTIAL, BUT NOT
OXIDATIVE PHOSPHORYLATION

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Trypanosoma brucei is a parasitic protist responsible for sleeping sickness in humans. The procyclic form of this parasite, transmitted by tsetse flies, is considered to be dependent on oxidative phosphorylation for ATP production. Indeed, its respiration was 55% inhibited by oligomycin, which is the most specific inhibitor of the mitochondrial F$_1$/F$_0$-ATP synthase. However, a 10-fold excess of this compound did not significantly affect the intracellular ATP concentration and the doubling time of the parasite was only 1.5-fold increased, suggesting that oxidative phosphorylation is not essential for procyclic trypanosomes. To further investigate the sites of ATP production, we studied the role of two ATP producing enzymes, which are involved in the synthesis of pyruvate from phosphoenolpyruvate: the glycosomal pyruvate phosphate dikinase (PPDK) and the cytosolic pyruvate kinase (PYK). The parasite was not affected by PPDK gene knockout. In contrast, inhibition of PYK expression by RNA interference was lethal for these cells. In the absence of PYK activity, the intracellular ATP concentration was reduced by up to 2.3-fold, whereas the intracellular pyruvate concentration was not reduced. Furthermore, we show that this mutant cell line still excreted acetate from d-glucose metabolism, and both the wild type and mutant cell lines consumed pyruvate present in the growth medium with similar high rates, indicating that in the absence of PYK activity pyruvate is still present in the trypanosomes. We conclude that PYK is essential because of its ATP production, which implies that the cytosolic substrate level phosphorylation is essential for the growth of procyclic trypanosomes.

The trypanosomatids are a group of parasitic protozoa of major medical and veterinary significance, including the human pathogens, Trypanosoma brucei, Trypanosoma cruzi, and Leishmania spp., which are responsible for sleeping sickness, Chagas disease, and leishmaniasis, respectively (1). Recently, an extremely powerful reverse genetic approach, i.e. RNA interference (RNAi), has been characterized in T. brucei (2) and adapted to constitutive and inducible expression in both the mammalian (bloodstream forms) and insect (procyclic form) stages of this parasite (3–7). RNAi has also been developed as a reverse genetic tool for another African trypanosome, Trypanosoma congolense, which infects livestock (8). Unfortunately, RNAi has not been shown to operate in Leishmania spp. (9) or in T. cruzi to date (2). In studying pathways shared by all of these trypanosomatids, such as those of carbohydrate metabolism, the T. brucei procyclic form constitutes an excellent model, thus conclusions drawn from RNAi experiments with these cells may also be applicable to the other parasites.

The procyclic trypanosomes grown in the commonly used SDM-79/FCS medium use d-glucose and L-threonine as major carbon sources, whereas, L-proline and L-glutamine are moderately consumed (10). L-Threonine is converted into equimolar amounts of excreted glycine and acetate, in a pathway involving acetyl-CoA as an intermediate metabolite (10, 11). d-Glucose catabolism is more elaborate as exemplified by the end products excreted, which include acetate, succinate, L-alanine, lactate, and carbon dioxide (CO$_2$) (12, 13). Most of the enzymes involved in the conversion of d-glucose into pyruvate are located in glycolyses (peroxisome-like organelles) (14, 15). Because of the apparent absence of pyruvate kinase (PYK) activity, it was originally proposed that pyruvate is produced from cytosolic PEP by the glycosomal PEP carboxykinase, the glycosomal malate dehydrogenase, and the cytosolic malic enzyme (see Fig. 1). In this scheme, pyruvate is then converted in the mitochondrion, to acetyl-CoA, which feeds the tricarboxylic acid cycle. In 1998, Van Hellemont et al. (16) showed that acetyl-CoA can also be converted into the excreted acetate by mitochondrial acetate:succinate-CoA transferase, which involves a succinate/succinyl-CoA cycle, thereby generating ATP by the mitochondrial succinyl-CoA synthetase (16) (Fig. 1). A possible role for NADH-dependent fumarate reductase in the production of the excreted succinate was controversial (17, 18).

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1 The abbreviations used are: RNAi, RNA interference; FCS, fetal calf serum; PYK, pyruvate kinase; PEP, phosphoenolpyruvate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; PEPCK, phosphoenolpyruvate carboxykinase; PPDK, pyruvate phosphate dikinase; UTR, untranslated region; PBS, phosphate-buffered saline; SHAM, salicylhydroxamic acid; MOPS,4-morpholinepropanesulfonic acid.

2 J. E. Donelson, personal communication.
FIG. 1. Schematic representation of the D-glucose, L-proline, L-threonine, L-glutamine, and pyruvate metabolisms in the procyclic form of *T. brucei*. The carbon sources used by the procyclic cells grown under standard conditions, i.e. SDM-79/FCS medium, are bold and boxed on a gray background. The number above or below each carbon source represents the percentage by which it, among all carbon sources, is consumed.
However, it was commonly assumed that succinate is a by-product of the mitochondrial tricarboxylic acid cycle (12, 13). Similarly, CO$_2$ was considered to be primarily produced by the tricarboxylic acid cycle (12, 17). L-Alanine seems to be produced during the midlog phase (data not shown). Excreted products (acetate, L-alanine, succinate, L-glycine, and CO$_2$) are in sodium carbonate, penicillin (100 units/ml) Similarly, CO$_2$ was considered to be primarily produced by the product of the mitochondrial tricarboxylic acid cycle (12, 13). T. brucei origin of lactate is not clear. Notwithstanding the debate on end energy metabolism in procyclic mitochondrion by substrate level phosphorylation and oxidative phosphorylation are targeted for RNAi (pyruvate kinase) and gene knockout (pyruvate phosphate dikinase). The ATP molecules produced in the cytosol and the ATP Production in Procyclic T. brucei

EXPERIMENTAL PROCEDURES

Cell Cultures—The human microglial cell line CHME-5 was cultured as a monolayer in 100-mm diameter Petri dishes, in minimal essential medium, supplemented with 20 mM glucose, 45 mM glutamine, 45 mM sodium carbonate, penicillin (100 units/ml$^{-1}$), streptomycin (0.1 mg/ml$^{-1}$), and 10% heat-inactivated fetal calf serum (24). Cultures were carried out for 5 days in a water-saturated incubator with a 9% CO$_2$ atmosphere. For the respiratory experiments microglia growth was performed on collagen beads (Cultispher-GL, Percell Biotica AB, Sweden). The cell suspension and the beads were mixed at least 24 h before the test, at a ratio of 3–5 $\times$ 10$^5$ cells per ml of beads. The procyclic form of T. brucei EATRO1125 was cultured at 27°C in SDM-79 medium containing 10% (v/v) heat-inactivated fetal calf serum, 0.5% low-hemin (SDM-79/FCS) (25). Alternatively, cells were grown in a modified version of the SDM-79/FCS medium, containing 1.6 mM L-glutamine, 4 mM pyruvate, 11 mM d-glucose, and 8 mM L-threonine.

*Inactivation of the ppdk Gene by Gene Knockout*—To construct the pL1ppdk plasmids, the DNA fragments containing the hygromycin resistance gene and the T7 RNA polymerase gene of the pHDS28 plasmid (26) were isolated by EcoRI and BamHI digestion of the pHDS28 plasmid and ligated into pBluescript SK$^+$ (Stratagene). The DNA fragments containing the pyruvate kinase gene (5’- and 3’-untranslated regions deleted; the ppdk gene (5’- and 3’-UTR) (27). The 5’-UTR fragment (661 bp) was generated by PCR using as primers the ppdk-5’ (5’-ATGGCCGGCGGCGCCCAAGGCAACTGACGAAAA-3’; the NotI restriction site is underlined) and ppdk-3’-5’ (5’-CAGCTGGCGCGGCGGCTGATTCTTTCACCCCGAG-3’; the Stul restriction site is underlined) oligonucleotides and as template the Cos8 genomic cosmid, which contains the cloned ppdk gene and its flanking regions (27). The NotI/BsmHII-digested PCR fragment was cloned into the NotI/MluI-digested pHDS28 plasmid (BssHI and MluI have compatible ends), to generate the pH5S plasmid. The 3’-UTR fragment (860 bp) was generated by PCR using as template the Cos8 cosmid and as primers the ppdk-5’ (5’-CACTAGCTAGCCGGCGGCGGCGGCTGATTCTTTCACCCCGAG-3’; the Stul restriction site is underlined) and ppdk-3’ (5’-CACTAGCTAGCCGGCGGCGGCGGCTGATTCTTTCACCCCGAG-3’; the NotI restriction site is underlined) oligonucleotides. The Stul/NheI-digested PCR fragment was cloned into Stul/NheI-digested pH5S plasmid to produce the pL1ppdk plasmid. The pL2ppdk plasmid was generated by replacing the XhoI/Stul fragment of the pL1ppdk plasmid, which encodes the hygromycin resistance gene and the T7 RNA polymerase gene, by the XhoI/Stul fragment of the pLew114 plasmid (27), which encodes the neomycin resistance gene and the tetracycline repressor gene under the control of the T7 RNA polymerase promoter. The pL1ppdk and pL2ppdk plasmids were linearized by NotI prior to parasite transformation. The procyclic EATRO1125 strain was sequentially transfected with the NotI-digested pL1ppdk and pL2ppdk plasmids and selection of hygromycin-resistant and hygromycin/neo-mycin-resistant clones, respectively, was performed as previously reported (4, 22).

Expression of an Anti-PYK Double Stranded RNA—To inhibit PYK expression (4), we generated in the pLew79 expression vector (kindly provided by E. Witting and G. A. M. Cross) (23) a “sense antisense” cassette that specifically targets the PYK gene (28) (Fig. 2). The 3’-end of the PYK gene was targeted, from position 1088 to 1519 bp (the ATG start and stop codons, of the PYK gene, are at positions 1 and 1500 bp, respectively) (28). The plasmid construction was performed as previously described (4, 21). Briefly, a PCR-amplified 484 bp containing the antisense sequence (431 bp of targeted sequence plus 42 bp used as a spacer between the sense and antisense sequences) was inserted in the HindIII and BamHI restriction sites of the pLew79 plasmid. Then a PCR-amplified 442 bp fragment containing the sense sequence was inserted, upstream of the antisense sequence, in HindIII and Xhol restriction sites (XhoI was introduced at the 3′ extremity of the anti-sense PCR fragment). The resulting plasmid, pLew79-ΔPYK, contained during the midlog phase (data not shown). Excreted products (acetate, L-alanine, succinate, L-glycine, and CO$_2$) are in white characters on a black background. The enzymatic reaction(s) leading to the production of lactate (from pyruvate and/or α-ketoglutarate), acetate (from L-threonine), and glutamate (from L-glutamine) are not indicated and are indicated by a question mark. The metabolic flux at each enzymatic step is tentatively represented by arrows with different thickess. Dashed arrows indicate steps that are supposed to occur at a background level or not at all, under the standard growth conditions. The glycolosomal and mitochondrial compartments are indicated and the boxed numbers represent enzymes targeted for RNAi (pyruvate kinase) and gene knockout (pyruvate phosphate dikinase). The ATP molecules produced in the cytosol and the mitochondrial by substrate level phosphorylation and oxidative phosphorylation are *bold* and *boxed*. AA, amino acid; AOB, amino oxobutyrate; 1,3BPG, 1,3-bisphosphoglycerate; C, cytochrome c; CoASH, coenzyme A; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; G-3-P, glyceraldehyde 3-phosphate; GLU, glutamate; 2Ket, 2-ketoglutarate; OA, 2-oxoacid; Oxal, oxalacetate; 3-PGA, 3-phosphoglycerate; PI, inorganic phosphate; PPi, inorganic pyrophosphate; yGlu, glutamate γ-semialdehyde; SucCoA, succinyl-CoA; UQ, ubiquinone pool. The enzymes are: 1, hexokinase; 2, glucose-6-phosphate isomerase; 3, fructose-6-phosphate kinase; 4, aldolase; 5, triose-phosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, phosphoglycerate kinase; 8, phosphoglycerate mutase; 9, enolase; 10, phosphoenolpyruvate carboxykinase; 11, malate dehydrogenase; 12, fumarase; 13, NADH-dependent fumarate reductase; 14, glyceral-3-phosphate dehydrogenase; 15, pyruvate kinase; 16, pyruvate phosphate dikinase; 17, malic enzyme; 18, alanine aminotransferase; 19, pyruvate dehydrogenase complex; 20, aspartate/succinyl-CoA transferase; 21, succinyl-CoA synthetase; 22, citrate synthase; 23, aconitase complex; 26, succinate dehydrogenase; 27, proline dehydrogenase; 28, succinyl-CoA dehydrogenase; 29, glycerine aminotransferase; 30, glutamate dehydrogenase; 31, L-threonine dehydrogenase; 32, acetate-CoA:cycteine S-carboxyl methyltransferase; 33, homoorotic inhibitory mutant of the activity of the glutamate dehydrogenase; 34, glycerol-3-phosphate oxidase; 35, alternative oxidase; 36, F$_0$/F$_1$-ATP synthase; I, II, III, and IV, complexes of the respiratory chain.
a chimeric construct composed of the sense and antisense version of a PYK gene fragment, separated by a 42-bp fragment, under the control of the tetracycline-inducible procyclin (PAPR) promoter (27) (Fig. 2). The procyclin EATRO1125.T7T and Δ1.2ppdk cell lines, which express the tetracycline repressor (4, 27), were transfected with the NotI-digested pLew79-ΔPYK plasmid and selection of phleomycin-resistant cells was performed as previously reported (4, 22).

Western Blot Analysis—Total protein extracts of the procyclic form of T. brucei (107 cells) were separated by SDS-PAGE (8%) and immunoblotted on Immobilon-P filters (Millipore) (29). Immunodetection was performed as described (29, 30) using as primary antibodies, the monoclonal mouse anti-PPDK (H9112) undiluted and the rabbit anti-glycero-

3-phosphate dehydrogenase diluted 1:100 (22, 31), and as secondary antibodies, anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), respectively.

Nuclear Magnetic Resonance (NMR) Experiments—4 × 106 T. brucei procyclic cells were collected by centrifugation at 1,400 × g for 10 min, washed once in PBS buffer, and incubated in 10 ml of incubation buffer (PBS buffer supplemented with 24 mM NaHCO3, pH 7.3) containing 110 μmol of D-[1-13C]glucose (11 mM) for 90–180 min at 27 °C. N-Glucose concentration in the medium was determined with the D-glucose Trinder kit (Sigma). The integrity of the cells during the incubation was checked by microscopic observation. After centrifugation for 10 min at 1,400 × g, the supernatant was lyophilized, re-dissolved in 500 μl of D2O, and 15 μl of pure dioxane was added as an external reference. 13C NMR spectra were collected at 125.77 MHz with a Bruker DPX500 spectrometer equipped with a 5-mm broad-band probe. Measurements were recorded at 25 °C under bivelir broad-band gated proton decoupling and D2O lock. Acquisition conditions were: 90° flip angle, 22,150 Hz spectral width, 64,000 memory size, and 21.5 s total recycle time. Measurements were performed overnight with 2,048 scans. Spectra were collected after a 1 Hz exponential line broadening. The specific 13C-enrichment of lactate (C3), acetate (C2), and succinate (C2 and C3) was determined from 1H-observed 13C-edited NMR (1H/13C NMR) spectra acquired under 13C-decoupling (32, 33). The sequence enabled the successive acquisitions of a first scan corresponding to a standard spin-echo experiment without any 13C-excitation and a second scan involving a 13C-inversion pulse. Subtraction of two alternate scans resulted in the editing of 1H spins coupled to 13C spins with a scalar coupling constant J1H,13C = 127 Hz. 13C-Decoupling during the acquisition collapsed the 13C-resonance to a single 1H resonance. Flip angles for rectangular pulses were carefully calibrated on both radiofrequency channels before each experiment. The relaxation delay was 5 s for a nearly complete longitudinal relaxation. The fractional 13C-enrichment at selected metabolite carbon positions was calculated as the ratio of the area of a given resonance in the 1H/13C NMR spectrum to its area in the standard spin-echo spectrum. The relative errors in the 13C-enrichment determinations were <5%. The amount of excreted products was calculated on the basis of the specific 13C-enrichment values and the carbon-13 content for each metabolite at the position of interest, using the 13C-enriched C1 glucose as quantitative reference.

Enzymatic Assays—Sonicated (5 s at 4 °C) crude extracts of trypanosomes suspended in cold hypotonic buffer (10 mM potassium phosphate, pH 7.8) were tested for enzymatic activities. PYK (34) and glycero-

3-phosphate dehydrogenase (GPDH) (31) activities were measured at 340 nm via reduction of NAD+ or oxidation of NADH, respectively, according to published procedures. The PYK activity was measured in the presence of its activator (fructose 2,6-bisphosphate) to increase the sensitivity of the assay, as described (34).

Determination of Metabolite Concentrations—The intracellular ATP, PEP, and/or pyruvate concentrations were determined on established procyclic cell lines in mid-log growth phase or CHME-5 human microglial cells grown on microbeads. Cell pellets (1–2 × 106 procyclic or 3–5 × 108 CHME-5 human microglial cells) were washed in cold PBS and frozen in liquid nitrogen. Lysis and deproteinization of the cellular pellets involved homogenization in 500 μl of cold perchloric acid (0.9 M) and neutralization (pH 6.5) by addition of KOH/MOPS (2.0 M). For ATP measurements, the firefly luciferase bioluminescence assay ("Quantitative ATP monitoring kit," ThermoLabsysystems) was used (35). Pyruvate and PEP concentrations were determined by enzymatic conversion into l-lactate, with the oxidation of stoichiometric amounts of NADH, as described before (36).

To determine the concentration of metabolites consumed or excreted by EATRO1125 procyclic trypanosomes, the inoculum (106 cells ml−1) was grown in the SDM-79/FCS medium containing 1.6 mM l-glutamine, 4 mM pyruvate, 11 mM α-glucose, and 8 mM l-threonine, until the stationary phase was reached. Aliquots of the growth medium were collected twice a day for the measurements. The quantity of α-glucose present in the medium was determined using the "glucose Trinder kit" (Sigma). Pyruvate concentration was determined enzymatically, as previously described (36). The concentration of the 20 amino acids present in the medium was determined by chromatography on an automatic amino acid analyzer coupled to a computing integrator (Beckman), after deproteinization of the samples by perchloric acid treatment.

Measurement of Oxygen Consumption—For oxygen consumption measurements, all cells were resuspended in their culture medium (including heat-inactivated fetal calf serum) at a density in the range of 1–2 × 106 cells/ml (CHME-5 human microglial cells) or 0.5–2 × 109 cells/ml (T. brucei procyclic form). Oxygen uptake was measured polarographically with a Clark type electrode (final volumes of 1 or 2
ml). Respiration rates were calculated with respect to calibration of the oxygen electrode with air-saturated medium containing 240 μM O2 at 25 °C (T. brucei procyclic form) or 220 μM O2 at 37 °C (CHME-5 human microglial cells). The effects of the addition of salicylhydroxamic acid (SHAM) (0.5 mM), KCN (1 mM), carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (0.1 mM), and oligomycin (from 0.1 to 5 μg/ml) were tested.

RESULTS

Oxidative Phosphorylation Is Not Essential for Procyclic Trypanosomes—To study the mitochondrial activity of procyclic T. brucei cells, we determined how oxygen consumption, ATP production, and cell growth were affected by effectors of mitochondrial metabolism (SHAM, KCN, CCCP, and oligomycin). This analysis was also performed on the CHME-5 human microglial cells, which depend on oxidative phosphorylation for ATP production (24). SHAM, KCN, and oligomycin are specific inhibitors of the alternative oxidase, cytochrome c oxidase, and F0/F1-ATP synthase, respectively, and CCCP is a protonophoric uncoupler that dissipates transmembrane proton gradients (37, 38). In eukaryotic cells the mitochondrial oligomycin-sensitive F0/F1-ATP synthase uses the proton gradient generated by the respiratory chain to produce ATP (39). This oxidative phosphorylation was illustrated here by the inhibition of cellular oxygen consumption upon the addition of an excess of oligomycin (40 and 78% inhibition in trypanosome and human cell lines, with 5 and 1.5 μg of oligomycin per ml protein, respectively) (Fig. 3A). This inhibition of respiration is because of an increase of the mitochondrial membrane proton gradient and confirms that the proton gradient generated by the respiratory chain is used by the F0/F1-ATP synthase to produce ATP in procyclic trypanosome as well as in human microglial cell lines (17, 24, 40). It is noteworthy that the oligomycin activity depends on the cell number (Fig. 3B). Indeed, 0.5 and 1.75 μg of

**Fig. 3.** Comparison of the respiratory characteristics of the T. brucei procyclic and human microglia cell lines. Panel A shows a representative assay of the effect of sequential addition of oligomycin (5 and 0.8 μg·mg⁻¹ protein for the trypanosome and human cells, respectively), CCCP (0.1 mM) and KCN (1 mM), to the EATRO1125 procyclic cells (10⁶ cells·ml⁻¹) and CHME-5 human microglia cells (2 × 10⁶ cells·ml⁻¹), resuspended in their growth medium containing FCS. Numbers along the traces represent the oxygen consumption rate expressed in nanomole of O₂ min⁻¹·10⁶ cells⁻¹ (underlined) and in nanomole of O₂ min⁻¹·mg⁻¹ protein (in brackets). In panel B, the oxygen consumption rate of the EATRO1125 procyclic cells (5 × 10⁷ or 2 × 10⁶ cells·ml⁻¹, corresponding to 0.2 and 0.8 mg of protein·ml⁻¹) resuspended in 1 ml of SDM-79/FCS medium was determined in the presence of increasing amounts of oligomycin. The values are expressed as a percentage of the ones observed in the absence of oligomycin (0.2 ± 0.05 nmol of O₂ consumed min⁻¹·10⁶ cells⁻¹). This analysis shows a direct correlation between the minimal amount of oligomycin required to completely inhibit the oligomycin-sensitive respiration and the number of cells.
ATP Production in Procyclic T. brucei

oligomycin inhibits completely the oligomycin-sensitive respiration of $5 \times 10^2$ and $2 \times 10^9$ T. brucei procyclic cells, which corresponds to 2.5 and 2.2 μg of oligomycin-mg$^{-1}$ protein, respectively. Addition of CCCP, after oligomycin treatment, induced a substantial increase in oxygen consumption, 2.8- and 5.7-fold for the trypanosome and human cells, respectively (Fig. 3A), because of the collapse of the proton gradient.

Respiration properties of procyclic trypanosomes and CHME-5 human microglial cells differ mainly by their sensitivity to SHAM (an inhibitor of mitochondrial alternative oxidase). Indeed, the oxygen consumption of the EATRO1125 procyclic cell line was inhibited by 60 ± 15% in the presence of 0.5 mM SHAM, whereas the human microglial cell line is insensitive to SHAM, because of the absence of an alternative oxidase in mammalian cells.

To determine the effect of mitochondrial effectors on energy transduction, the intracellular ATP concentration was measured periodically over 90 or 120 min of incubation (Fig. 4). In the absence of extracellular carbon sources, the intracellular ATP concentration collapsed after an hour of incubation for both procyclic trypanosomes and human microglia cells. Similarly, the intracellular concentration of ATP in the CHME-5 human microglial cells was considerably reduced after 60 min of incubation with 1 mM KCN (80%) or 5 μg of oligomycin-mg$^{-1}$ protein (60%) (Fig. 4A), which indicates that most of the ATP produced by this cell line depends on oxidative phosphorylation, as previously observed (24). In contrast, these mitochondrial effectors have little or no effect on the steady state ATP concentration in the EATRO1125 procyclic cells (Fig. 4B). Indeed, intracellular ATP was reduced by ~20% after 20 min of treatment with 1 mM KCN or 0.5 mM SHAM, and reduced by 35% in the presence of both KCN (1 mM) and SHAM (0.5 mM) (data not shown). More interestingly, no effect was observed following the addition of 6, 12, and 24 μg of oligomycin-mg$^{-1}$ protein (Fig. 4B), which corresponds to up to 10-fold the concentration that completely inhibits the oligomycin-sensitive respiration (Fig. 3B). Furthermore, equivalent oligomycin concentrations (1 to 12 μg-mg$^{-1}$ protein) only had a modest effect on the growth of the parasite (doubling time: 18 versus 12 h for the wild type cells) (Fig. 5). In the course of this 8-day experiment, the intracellular ATP concentration was not affected by oligomycin treatment (data not shown). A very large excess of oligomycin (~1 mg-mg$^{-1}$ protein) kills 100% of the procyclic trypanosomes after 1 day of treatment (data not shown), probably as a consequence of a nonspecific effect (41). It is noteworthy that the 35% reduction of intracellular ATP concentration, in the presence of SHAM and KCN, is probably the indirect consequence of the complete inhibition of all mitochondrial electron transport activity that appears to be essential for the parasite.

These data indicate that inhibition of the F$_{0}$/F$_{1}$-ATP synthase does not significantly affect ATP levels in procyclic trypanosomes. Other routes such as substrate level phosphorylation, may then be used to generate ATP. To test this hypothesis, we studied the role of two glycolytic enzymes that produce ATP, from PEP, in the glycosomes (PPDK (22)) or in the cytosol (PYK (21)) (see Fig. 1).

**PPDK Is Not Essential for Procyclic Cells**—PPDK is a glycosomal enzyme present in all trypanosomatids analyzed so far, which produces pyruvate, ATP, and inorganic phosphate from PEP, AMP, and pyrophosphate (22). To study the role of this enzyme, we replaced both PPDK alleles with two DNA fragments (pΔ1ppdk and pΔ2ppdk) through homologous recombination using the upstream and downstream PPDK UTRs flanking these fragments (Fig. 2). The EATRO1125 procyclic form of *T. brucei* was sequentially transfected with pΔ1ppdk and pΔ2ppdk to generate the Δ1ppdk (::HYG-TTRNAPol) and Δ1.2ppdk (::HYG-TTRNAPol::ppdk::NEO-TetR) cell lines. The Δ1ppdk cell line, which contains a single PPDK copy, showed a decrease of the PPDK signal by Western blot analysis, which is correlated with a 47% decrease of the PPDK activity, as compared with the wild type EATRO1125.777 cell line (Fig. 6). For the Δ1.2ppdk cell line, in which both PPDK alleles are deleted, no PPDK is detectable by activity assays nor by Western blot (Fig. 6) and immunofluorescence analyses (data not shown). The doubling time of the Δ1.2ppdk cell line is not affected (Fig. 7), indicating that PPDK is not essential for cell viability under the standard growth conditions. However, the rate of n-glucose consumption is slightly reduced in the Δ1.2ppdk cell line, as compared with the wild type cells (1.0 versus 1.3 μmol h$^{-1}$ mg$^{-1}$ protein), which may be an adaptation to the PPDK gene knockout.

To further study the role of PPDK in glucose metabolism of procyclic trypanosomes, we used carbon-13 NMR (13C NMR) spectroscopy to compare the metabolic end products excreted by the Δ1.2ppdk and untransfected cell lines fed with [1-13C]glucose. The parasites were incubated in PBS/NaHCO$_3$ medium containing 110 μmol of [1-13C]glucose, as the only carbon source, until 30–60 μmol of n-glucose were consumed by each cell line. The incubation medium was then analyzed by NMR spectroscopy. To determine the [13C] amount in each excreted end product, the analysis was performed on fully relaxed spectra. For each of the *T. brucei* procyclic cell lines (wild type and mutants) analyzed by this approach, we calculated that approximately two molecules are excreted (out of the products succinate, acetate, lactate, malate, and fumarate) per molecule of glucose consumed (21). For convenience, we have expressed the quantity of individual excreted molecules as the percentage of all of the excreted end products (Table I). The wild type procyclic form excretes mainly succinate (69.4% of all the excreted 13C-enriched molecules), acetate (19.4%), and lactate (9.5%), with traces of malate (1.3%) and fumarate (0.4%) (Fig. 8A and Table I). The 13C-enriched end products of glucose metabolism excreted by the wild type and Δ1.2ppdk cell lines are in the same range, except the mutant cell line Δ1.2ppdk, which shows a 3.8-fold reduction of lactate (9.5% versus 2.5%) (Table I). In conclusion, ppdk gene knockout does not significantly affect n-glucose metabolism nor the doubling time of the procyclic trypanosomes. This indicates that the involvement of PPDK in ATP production is not essential. However, in the absence of PPDK, another ATP producing activity (yet to be determined) may compensate for this loss in the Δ1.2ppdk cell line.

**PYK Is Essential in Procyclic Trypanosomes**—According to the model recently proposed (Fig. 1), PYK may play a role in glucose metabolism in procyclic cells (21). Indeed, we showed that a significant PYK activity is present in the cytosol of the procyclic form, suggesting that PYK can produce pyruvate from cytosolic PEP. To study the role of PYK, we inactivated the expression of its gene by RNAi. To inhibit PYK expression by RNAi in the procyclic form of *T. brucei*, the pLew79 vector (27) was used to express double stranded RNA molecules containing linked sense and antisense copies of the targeted PYK sequence, under control of a tetracycline-inducible promoter (Fig. 2). The recombinant plasmid (pLew79-ΔPYK) was inserted into the rDNA spacer of the EATRO1125.777 cell line expressing the tetracycline repressor (4, 27), to generate the ΔPYK cell line. The PYK activity and the doubling time of the tetracycline-induced (ΔPYK)i or non-induced (ΔPYKni) ΔPYK cell line were determined. The PYK activity of the ΔPYKni cell line is reduced as compared with the wild type trypanosomes (Fig. 7), whereas the double stranded RNA should not be expressed.

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in the absence of tetracycline. This is probably because of a leakage of the tetracycline-repressor control, as previously described (4, 27). Interestingly, the reduction of the PYK activity (2.3-fold) is directly correlated with the increase of the doubling time (2.4-fold) observed for the \( \text{H9004}_{\text{PYK}} \text{.ni} \) cell line (Fig. 7). The importance of PYK for cell growth was confirmed by inducing

FIG. 5. Growth of the \( \text{T. brucei} \) procyclic form in the presence of oligomycin. Growth of the EATRO1125 procyclic cell line was started with \( 3 \times 10^6 \) cells \( \text{ml}^{-1} \) SDM-79/FCS medium, in the presence or absence of oligomycin. The cell density was maintained between \( 3 \times 10^6 \) and \( 10^7 \) cells \( \text{ml}^{-1} \) to obtain relative concentrations of oligomycin in the range of 1–3, 2–6, or 4–12 \( \mu \text{g} \text{mg}^{-1} \) protein, depending on the experiment. Cumulative cell numbers reflect normalization for dilution during cultivation. The intracellular ATP concentration was determined along these experiments with values of 22.6 ± 3.5 nmol\( \text{mg}^{-1} \) protein (no oligomycin; 6 measurements) and 22.3 ± 2.8 nmol\( \text{mg}^{-1} \) protein (4–12 \( \mu \text{g} \) of oligomycin\( \text{mg}^{-1} \) protein; 5 measurements).

FIG. 6. Western blot analysis of the \( \Delta_{1 \text{ppdk}} \) and \( \Delta_{1.2 \text{ppdk}} \) procyclic cell lines. Lysates (10^7 cells) of the procyclic cell lines, EATRO1125 (WT), \( \Delta_{1 \text{ppdk}} \) (\( \Delta_1 \)), and \( \Delta_{1.2 \text{ppdk}} \) (\( \Delta_{1.2} \)), were analyzed by Western blot with the anti-PPDK and anti-glycerol-3-phosphate dehydrogenase (GPDH) immune sera. The PPDK activity of each cell line is shown under the blot. ND, not determined.

in the absence of tetracycline. This is probably because of a leakage of the tetracycline-repressor control, as previously described (4, 27). Interestingly, the reduction of the PYK activity (2.3-fold) is directly correlated with the increase of the doubling time (2.4-fold) observed for the \( \Delta_{\text{PYK.ni}} \) cell line (Fig. 7). The importance of PYK for cell growth was confirmed by inducing

**Fig. 4.** Effect of metabolic inhibitors on the steady state amount of intracellular ATP in human microglia cells (panel A) and procyclic \( \text{T. brucei} \) (panel B). Monolayer CHME-5 human microglia cells in Petri dishes (\( 4 \times 10^6 \) cells) were incubated at 37 °C in 10 ml of minimal essential medium in the absence or presence of oligomycin (1.5 \( \text{g} \text{ml}^{-1} \) or KCN (1 mM). The EATRO1125 procyclic cells (4 \( \times 10^7 \) cells\( \text{ml}^{-1} \)) were incubated at 27 °C in 10 ml of SDM-79/FCS medium in the absence or presence of oligomycin (1, 2, or 4 \( \mu \text{g} \text{ml}^{-1} \)), SHAM (0.5 mM), or KCN (1 mM). The intracellular amount of ATP was determined periodically in the cells from one Petri dish (panel A) or on 2-ml samples (panel B), as described under "Experimental Procedures." The cells incubated in PBS/dialyzed FCS (10%) showed an amount of intracellular ATP reduced by 90% after 90 min of incubation in the absence of carbon source, indicating that the intracellular ATP pool cannot be maintained, in the absence of extracellular carbon sources. However, for the human microglia cells (panel A), the intracellular ATP concentration did not change significantly during 40 min of incubation, suggesting the contribution of their high intracellular phosphocreatine content to maintain the ATP steady state, as described for this cell type in response to heat stress situation (24). It is noteworthy that the steady state amount of ATP is stable (as observed for the curve, \( \square \) when the same experiment is conducted on trypanosomes incubated in PBS/dialyzed FCS (10%) containing all the carbon sources (data not shown). The values represent the means of two to four independent experiments.

**Fig. 5.** Growth of the \( \text{T. brucei} \) procyclic form in the presence of oligomycin. Growth of the EATRO1125 procyclic cell line was started with \( 3 \times 10^6 \) cells\( \text{ml}^{-1} \) SDM-79/FCS medium, in the presence or absence of oligomycin. The cell density was maintained between \( 3 \times 10^6 \) and \( 10^7 \) cells\( \text{ml}^{-1} \) to obtain relative concentrations of oligomycin in the range of 1–3, 2–6, or 4–12 \( \mu \text{g} \text{mg}^{-1} \) protein, depending on the experiment. Cumulative cell numbers reflect normalization for dilution during cultivation. The intracellular ATP concentration was determined along these experiments with values of 22.6 ± 3.5 nmol\( \text{mg}^{-1} \) protein (no oligomycin; 6 measurements) and 22.3 ± 2.8 nmol\( \text{mg}^{-1} \) protein (4–12 \( \mu \text{g} \) of oligomycin\( \text{mg}^{-1} \) protein; 5 measurements).
in the exponential growth phase (between $10^5$ and $10^7$ cells with the EATRO1125.T7T procyclic cell lines. (PYK the expression of double stranded RNA with tetracycline products (pyruvate and ATP), we determined the effect of the observed (42).

cating that PYK is essential for cell viability, as previously days of tetracycline induction leads to cell death (Fig. 7), indi-

Fig. 7. Comparative analysis of PPDK and/or PYK mutants with the EATRO1125.T7T procyclic cell lines. The growth curves of the procyclic EATRO1125.T7T cell line and different mutant cell lines were determined in the SDM-79/FCS medium. Cells were maintained in the exponential growth phase (between $10^6$ and $10^7$ cells ml$^{-1}$) and cumulative cell numbers reflect normalization for dilution during cul-
tivation. The mutant cell lines $\Delta 1.2ppdk/\Delta PYK$ and $\Delta PYK$ were in-

bulated in the presence (i) or absence (ni) of tetracycline. The PYK activity (milliunit:mg$^{-1}$ protein) of each cell line is indicated in brackets, above the curves, in the upper panel. The arrow shows when the cells were collected, in different sets of experiments, to perform NMR analysis. In the case of cell lines grown in the presence of tetracycline ($\Delta 1.2ppdk/\Delta PYK$ i and $\Delta PYK$ i), the PYK activity decreases over time to become undetectable (ND), as indicated in the table of the lower panel (the PYK activities are normalized with the GPDH activity measured in the same samples).

the expression of double stranded RNA with tetracycline ($\Delta PYK$ i). The complete inhibition of PYK expression after 6 days of tetracycline induction leads to cell death (Fig. 7), indicat-
ing that PYK is essential for cell viability, as previously observed (42).

Because, PYK and PPDK may compete for the same sub-
strate (PEP) or be complementary because they have the same products (pyruvate and ATP), we determined the effect of the simultaneous loss of both enzymes. The $\Delta 1.2ppdk$ cell line, which expresses the tetracycline repressor, was transfected by the pLew79-$APYK$ plasmid to generate the $\Delta 1.2ppdk/\Delta PYK$ cell line. The death of the $\Delta 1.2ppdk/\Delta PYK$ i trypanosomes, 6 days after tetracycline induction, correlated with the loss of the PYK activity, as observed for the $\Delta PYK$ i cell line (Fig. 7). We also observed a reduction of the PYK activity in the non-induced $\Delta 1.2ppdk/\Delta PYK$ cell line ($\Delta 1.2ppdk/\Delta PYK$ ni) as compared with the wild type cells. However, this substantial reduction of the PYK activity (3-fold) was associated with only a small increase in the doubling time (1.3-fold), as opposed to the $\Delta PYK$ i cell line (Fig. 7). Comparison of the $\Delta 1.2ppdk/\Delta PYK$ ni and $\Delta PYK$ ni cell lines, shows that the procyclic cells are less af-
fected by the reduction of the PYK activity, when PPDK is not expressed. This indicates that the absence of PPDK compens-
sates for the decrease of the PYK activity in the $\Delta 1.2ppdk/\Delta PYK$ ni mutant. Because PYK and PPDK may compete for the same PEP substrate, these data could suggest that the meta-

bolic flux through the PYK pathway is increased in the absence of PPDK.

ATP Produced by PYK Is Essential for Procyclic Cells—PYK could be essential either for its role in ATP production or in pyruvate production. To distinguish which of these roles were essential, we conducted three different analyses. First, the metabolic end products excreted by the tetracycline-induced ($\Delta PYK$ i and $\Delta 1.2ppdk/\Delta PYK$ i) or non-induced ($\Delta PYK$ ni and $\Delta 1.2ppdk/\Delta PYK$ ni) $\Delta PYK$ and $\Delta 1.2ppdk/\Delta PYK$ cell lines, fed with $\delta$-[1-$^{13}$C]glucose as the only carbon source, were moni-
tored by NMR spectroscopy, as described above (Fig. 8B and Table I). For the tetracycline-induced cells, this NMR analysis was conducted 5 days after addition of tetracycline, when the PYK activity reached a value $< 1$ milliunit:mg$^{-1}$ protein. At this stage, the rate of $\delta$-glucose consumption is not affected by the inhibition of PYK expression. Interestingly, the amount of excreted $^{13}$C-enriched acetate is only reduced by a small amount in the absence of PYK activity (13.9% $\pm$ 0.1 and 12.2$\%$ $\pm$ 0.05 for the $\Delta PYK$ i and $\Delta 1.2ppdk/\Delta PYK$ i cell lines, respectively, versus 19.4% $\pm$ 1.2 for the wild type cells) (Table I). This implies that $^{13}$C[pyruvate is produced, from $\delta$-[1-$^{13}$C]glucose, in the mutant cell lines. Indeed, under our exper-
imental conditions (where $\delta$-[1-$^{13}$C]glucose is the only carbon source), excreted $^{13}$C[acetate is produced from $\delta$-pyruvate by the pyruvate dehydrogenase complex and acetate:succinyl-
CoA transferase (see Fig. 1). In the absence of detectable PYK activity, succinate excretion is only slightly increased, whereas the amounts of excreted malate and fumarate are increased $\sim 10$- and 4-fold, respectively (Table I), in both the $\Delta PYK$ i and $\Delta 1.2ppdk/\Delta PYK$ i cell lines. This NMR analysis suggests that, in the absence of PYK, the glycolytic flux is probably reoriented through PEPC, which leads to an increase in intracellular malate concentration. Approximately half of the malate is con-
verted to pyruvate by the malic enzyme. Pyruvate is then converted to acetate that is excreted along with the remaining half of the malate (13.9$\%$ $\pm$ 0.1 excreted acetate versus 13.9$\%$ $\pm$ 0.8 excreted malate in the $\Delta PYK$ i cell line and 12.2$\%$ $\pm$ 0.05 versus 12.6$\%$ $\pm$ 0.4 in the $\Delta 1.2ppdk/\Delta PYK$ i cell lines) (Table I).

Second, we observed that pyruvate (1 mm), as well as t-

threonine (2.5 mm) and l-glutamine (0.46 mm) present in the SDM-79/FCS medium commonly used for cultivation of the parasites, are rapidly consumed. The SDM-79/FCS medium was modified by increasing the pyruvate (4 mm final), l-glutamine (1.6 mm final), and l-threonine (8 mm final) concentra-
tions (p-glucose concentration was also increased up to 11 mm). In the mid-log phase, the EATRO1125.T7T cell line consumes 0.8 mmol of pyruvate$^{-1}$ h$^{-1}$ protein$^{-1}$. Interestingly, pyruvate is one of the major carbon sources present in the growth me-
dium. Indeed, only $\delta$-glucose (1.4 mmol$h^{-1}$ mg$^{-1}$ protein) and l-threonine (1.25 mmol$h^{-1}$ mg$^{-1}$ protein) showed a higher con-
sumption rate, whereas l-proline (0.45 mmol$h^{-1}$ mg$^{-1}$ protein) and l-glutamine (0.4 mmol$h^{-1}$ mg$^{-1}$ protein) are consumed with a lower rate (data not shown). Interestingly, the rate of pyruvate consumption is not significantly affected by arrest of PYK expression in the tetracycline-induced and non-induced $\Delta 1.2ppdk/\Delta PYK$ (0.6 and 1.1 mmol$h^{-1}$ mg$^{-1}$ protein, before and 3 days after induction, respectively) and $\Delta PYK$ (0.65 and 1.25 mmol$h^{-1}$ mg$^{-1}$ protein, before and 3 days after induction, respectively) cell lines. Similarly, the rate of l-proline con-
sumption is not affected by inhibition of PYK expression (data
Here, we confirm the previous data and we show for the first time, by using modern metabolic techniques, that the parasite primarily consumes D-glucose and L-threonine as carbon sources, and to a lower extent L-proline and L-glutamine (25). D-Glucose metabolism has received more attention than the metabolism of the other carbon sources. When grown in SDM-79, or equivalent media, this insect stage of T. brucei produces up to 60% and 14% of its ATP from D-glucose and L-threonine, respectively. This is in contrast to the procyclic form, where D-glucose is metabolized to a much lesser extent (25).

Carbon-13 NMR spectra of procyclic T. brucei. To perform this NMR analysis, the EATRO1125.T7T cell lines were incubated with D-[1-13C]glucose. To determine the position of the enriched [13C] in each detected molecule is given, as obtained by a quantitative analysis of NMR spectra. The values represent the mean ± S.D. deduced from three to five NMR spectra.

| Succinate | Acetate | Lactate | Malate | Fumarate |
|----------|--------|--------|--------|----------|
| EATRO1125.T7T | 69.4 ± 1.5 | 19.4 ± 1.2 | 9.5 ± 0.8 | 1.3 ± 0.3 | 0.4 ± 0.02 |
| ΔPPDK | 77.5 ± 1.5 | 17.5 ± 0.2 | 2.5 ± 0.2 | 2.1 ± 0.3 | 0.55 ± 0.05 |
| ΔPYK | 78.0 ± 0.3 | 15.2 ± 0.07 | 2.4 ± 0.2 | 3.8 ± 0.3 | 0.5 ± 0.5 |
| ΔPYK.i | 74.3 ± 0.5 | 13.9 ± 0.1 | 0.6 ± 0.1 | 13.9 ± 0.8 | 1.6 ± 0.3 |
| Δ1.2ppdk/ΔPYK | 79.0 ± 1 | 15.9 ± 0.2 | 1.1 ± 0.1 | 4.3 ± 0.2 | 0.62 ± 0.02 |
| Δ1.2ppdk/ΔPYK.i | 73.4 ± 0.4 | 12.2 ± 0.05 | 0.12 ± 0.03 | 12.6 ± 0.4 | 1.8 ± 0.2 |

**DISCUSSION**

In 1979, Brun and Schönberger (25) developed a semi-defined medium, called SDM-79, which became the most popular medium for growing the procyclic form of T. brucei (25). When grown in SDM-79, or equivalent media, this insect stage parasite primarily consumes D-glucose and L-threonine as carbon sources, and to a lower extent L-proline and L-glutamine (Ref. 10, see Fig. 1). D-Glucose metabolism has received more attention than the metabolism of the other carbon sources. Here, we confirm the previous data and we show for the first time...
time, that pyruvate is also consumed with a rate higher than observed for L-proline (0.8 versus 0.45 mmol/h/g g protein).

Three cytosolic or mitochondrial enzymatic activities of D-glucose metabolism produce ATP by phosphorylation at the substrate level, i.e. phosphoglycerate kinase, PYK, and succinyl-CoA synthetase (steps 7, 15, and 21 in Fig. 1). The recent development in T. brucei of an RNAi approach permitting conditional gene expression (2–7), provides an excellent tool to study genes potentially essential for cell viability, such as phosphoglycerate kinase, PYK, and succinyl-CoA synthetase. Inactivation of phosphoglycerate kinase gene expression would probably not be informative in determining whether it plays an essential role in ATP generation, because phosphoglycerate kinase catalyzes the last step of both D-glucose and L-proline metabolism, is essential for the procyclic cells (20, 23), and the same has been proposed for nucleotides (AMP, ADP, and ATP) and NAD"/NADH (12–15, 17). Our data suggest that one or more alternative routes are used to regenerate the ATP consumed by the first glycolytic steps (steps 1 and 3 in Fig. 1), or that the glycosomal nucleotide pool is exchanged with other subcellular compartments. Detailed analyses of mutants, including the PEPCK mutant, will be helpful in understanding how the ATP/ADP, as well as the NAD"/NADH, balances are conserved in the glycosomes of these parasites.

We showed that the production of ATP by oxidative phosphorylation is not essential for the procyclic cells. Indeed, inhibition of the F$_0$/F$_1$-ATP synthase (step 36 in Fig. 1), by a very specific inhibitor (oligomycin), does not affect the intracellular steady state concentration of ATP and only moderately affects the doubling time of the parasite (18 versus 12 h). However, inhibition of cytochrome oxidase by 1 mM cyanide (IV in Fig. 1) considerably affects the doubling time of the procyclic cells (23), and addition of both 1 mM cyanide and 0.5 mM SHAM (a specific inhibitor of the alternative oxidase, step 35 in Fig. 1), kills all parasites within 2 days of incubation (data not shown), indicating that the mitochondrial electron transport chains (cyanide-sensitive and SHAM-sensitive) are essential for this parasite. An important question, which has not yet been completely answered, is what is(are) the role(s) of these mitochondrial electron transport chains in procyclic T. brucei? As in most eukaryotes, a primary role is probably reoxidizing reduced equivalents, such as NADH produced in the mitochondrion during the metabolism of D-glucose/pyruvate (step 19 in Fig. 1), l-threonine (step 31), and l-proline/l-glutamine (steps 25, 28, and 30), as well as that of other substrates. The other essential role of the cyanide-sensitive respiratory chain is to generate a transmembrane proton gradient, which is used for essential mitochondrial functions, such as ion (Ca$^{2+}$) regulation and the mitochondrial protein and metabolite import. The proton gradient can also be used by the F$_0$/F$_1$-ATP synthase to produce ATP by oxidative phosphorylation, however, our data strongly suggest that this is not essential for the procyclic T. brucei. Thus, it appears that, in procyclic trypanosomes, production of ATP is not the primary role of the mitochondrial electron transport chains. It is noteworthy that the bloodstream forms of this parasite lack a full respiratory chain and use the F$_0$/F$_1$-ATP synthase to generate the transmembrane proton gradient from ATP hydrolysis (41). As discussed above, procyclic trypanosomes use the electron chain transport to generate the transmembrane proton gradient. The role of the
F$_0$/F$_1$-ATP synthase in mitochondrial ATP metabolism of the procyclic form of *T. brucei* therefore remains uncertain. The generation and analysis of RNAi mutants affecting expression of F$_0$/F$_1$-ATP synthase subunits, will be helpful to answer this question.

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ATP Generation in the *Trypanosoma brucei* Procyclic Form: CYTOSOLIC SUBSTRATE LEVEL PHOSPHORYLATION IS ESSENTIAL, BUT NOT OXIDATIVE PHOSPHORYLATION

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