Proline metabolism has been studied in procyclic form Trypanosoma brucei. These parasites consume six times more proline from the medium when glucose is in limiting supply than when this carbohydrate is present as an abundant energy source. The sensitivity of procyclic T. brucei to oligomycin increases by three orders of magnitude when the parasites are obliged to catabolize proline in medium depleted in glucose. This indicates that oxidative phosphorylation is far more important to energy metabolism in this latter case than when glucose is available and the energy needs of the parasite can be fulfilled by substrate level phosphorylation alone. A gene encoding proline dehydrogenase, the first enzyme of the proline catabolic pathway, was cloned. RNA interference studies revealed the loss of this activity to be conditionally lethal. Proline dehydrogenase defective parasites grew as wild-type when glucose was available, but, unlike wild-type cells, they failed to proliferate using proline. In parasites grown in the presence of glucose, proline dehydrogenase activity was markedly lower than when glucose was absent from the medium. Proline uptake too was shown to be diminished when glucose was abundant in the growth medium. Wild-type cells were sensitive to 2-deoxy-D-glucose if grown using glucose was abundant in the growth medium. Wild-type Proline uptake too was shown to be diminished when glucose, proline dehydrogenase activity was markedly but, unlike wild-type cells, they failed to proliferate parasites grew as wild-type when glucose was available, conditionally lethal. Proline dehydrogenase defective parasites grew as wild-type when glucose was available, but, unlike wild-type cells, they failed to proliferate using proline. In parasites grown in the presence of glucose, proline dehydrogenase activity was markedly lower than when glucose was absent from the medium. Proline uptake too was shown to be diminished when glucose was abundant in the growth medium. Wild-type cells were sensitive to 2-deoxy-D-glucose if grown using glucose as the principal carbon source, but not in glucose-rich medium, indicating that this non-catabolizable glucose analogue might also stimulate repression of proline utilization. These results indicate that the ability of trypanosomes to use proline as an energy source can be regulated depending upon the availability of glucose.

African trypanosomes of the brucei subgroup are responsible for a number of important diseases in man and animals (1). The metabolism of these organisms has been the subject of considerable interest. Bloodstream form trypanosomes are entirely dependent upon glycolytic substrate level phosphorylation for the generation of energy (2, 3). The first seven steps of the glycolytic pathway are localized to an unusual organelle, the glycosome (4). A glycerol 3-phosphate:dihydroxyacetone-phosphate shuttle operates between the glycosome and the mitochondrion where a plant like ubiquinone-linked alternative oxidase acts to transfer electrons to oxygen (5). This shuttle is critical in maintaining the NAD+/NADH balance within the glycosome. No net ATP synthesis occurs in the glycosome but ATP is produced cytosolically by the pyruvate kinase reaction (6). Pyruvate is excreted from these cells with no further metabolism and a mere two moles of ATP are synthesized per mole of glucose used. Bloodstream form trypanosomes can sustain this profligate metabolism as they are exposed to a steady supply of high glucose in mammalian blood. They cannot, however, use non-carbohydrate substrates for the generation of energy. Glucose metabolism is thus perceived as an excellent target for therapeutic intervention in bloodstream form trypanosomes (7). It appears that the Trypanosoma brucei bloodstream energy metabolism is very simple and contrasts with the more elaborate version present in all the other trypanosomatids analyzed so far, including the insect stages of T. brucei (3, 8).

The life cycle of brucei group trypanosomes is complex. Multiple distinct stages exist in the tsetse fly vector that carries the parasite between mammalian hosts (9). The metabolism of the parasites, as they proliferate in the midgut of their tsetse fly vector, is markedly different from that used by the bloodstream form. In the tsetse midgut, glucose is scarce but may become transiently abundant following insect blood meals. Proline is a key energy source within the tsetse fly (10, 11), and it has been speculated that this is a main energy source for the procyclic form trypanosomes too (12–14). However, procyclics make efficient use of glucose, which is the preferred carbon source in the glucose-rich medium commonly used to grow these parasites (15, 16). Until recently, it was widely accepted that procyclic trypanosomes produce ATP primarily by the mitochondrial F$_o$/F$_i$-ATP synthase (oxidative phosphorylation) exploiting the proton gradient across the mitochondrial inner membrane generated by the respiratory chain (5). In addition, the respiratory chain was considered to be fed chiefly by NADH produced by the tricarboxylic acid cycle (6). However, recent publications questioned these conclusions and a new model has been proposed (17) (Fig. 1). A functional Krebs cycle is not essential for energy metabolism and aconitase-defective cells thrive using glucose as an energy source (18). Moreover, blocking the mitochondrial F$_o$/F$_i$-ATP synthase has little impact on growth and does not affect ATP production in glucose-rich medium (19), while substrate level phosphorylation does appear to be essential to growth (19, 20). Essential sites of ATP production by substrate level phosphorylation include the cytosol (phospho-
glycerate kinase and pyruvate kinase) and the mitochondrion (sucncyl-CoA synthetase). Interestingly, the succinyl-CoA synthetase, shown to be essential to procyclic trypanosomes, catalyzes the last step of both glucose and proline degradation to produce the excreted end products acate and succinate, respctively (Fig. 1) (18, 21).

To date, no in vitro studies using cells grown in glucose-depleted medium, conditions to mimic the midgut environment of tsetse flies between blood meals, appear to have been conducted. We therefore set out to investigate the ability of procyclic trypanosomes to catabolize proline and to determine whether the availability of glucose influences this. To address this question, we adapted two T. brucei procyclic strains (EATRO1125 and 427) to glucose-depleted medium and studied their carbon source consumption and sensitivity to oligomycin (the most specific inhibitor of the F0/F1-ATP synthase). In addition, we have generated and analyzed a mutant cell line inhibited for the second step of the proline metabolism (proline
dehydrogenase (PRODH)). This analysis shows that glucose exerts a negative control on proline metabolism by down-regulating PRODH and affecting proline uptake (Fig. 1). EXPERIMENTAL PROCEDURES Trypanosoma and Cell Culture—T. brucei strain 427 and EATRO1125 procyclic forms were cultivated at 25 °C in SDM79 medium (16) or a glucose-depleted medium derived from SDM79, called SDM79 supplemented with 10% (v/v) fetal calf serum and 25% (v/v) conditioned medium collected and filtered after cultivation of wild-type parasites. Cultures of one clone (strain 427), washed, and resuspended in Zimmerman’s post-fusion medium by centrifuging at 3000 × g of protein solution at 1 mg ml−1 was determined using the Alamar blue assay (36). Parasite density was measured every 24 h with an improved light path at 600 nM.

RNA Interference (RNAi)—A 606-bp, XbaI-flanked, fragment of the PRODH gene was amplified by PCR from T. brucei cDNA using specific primers each containing an XbaI restriction site (5′-ATTTCGCAACCCCTCACTGCTTATGTTTATTG3′). The amplification was performed on the Biocad 700E work station using Ni2+ chelate chromatography (Novagen) with the elution buffer recommended by the manufacturer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 500 mM imidazole). Antiserum was raised in rabbits (Diagnostics Scotland), with an initial injection of 0.4 ml of protein solution at 1 mg ml−1 followed by three more 0.2-ml injections at 1-month intervals. Antibodies were purified using an AminoLink® ( Pierce) kit carrying immobilized purified PRODH on a solid support. Purified antibodies were aliquoted and stored at −20 °C until use. Western blotting of SDS-PAGE separated proteins on nitrocellulose membranes involved blocking for 3 h at room temperature with 1× TBS (0.02 mM Tris-HCl, 13.7 mM NaCl) containing 5% (v/v) powdered milk and 0.2% (v/v) Tween 20. The primary antibody, anti-PRODH (rabbit polyclonal antibody diluted 1:2000) or specific PRODH antibody (diluted 1:500 to 1:5000), was made up in 1× TBS, 1% (v/v) powdered milk, and 0.1% (v/v) Tween 20, before being added to the membrane and shaken overnight at 4 °C. The SuperSignal chemiluminescent substrate (Pierce) protocol with 1:2000 diluted anti-mouse IgG conjugated to peroxidase or with 1:500 to 1:5000 anti-rabbit horseradish peroxidase-coupled IgG (Sigma) allowed the detection of the proteins according to manufacturer’s specifications.

Proline Dehydrogenase Assay—PRODH activity was measured following the reduction of the electron-accepting dye iodonitrophenyl tetrazolium (INT), at 520 nm (30), or dichlorophenolindophenol (DCPIP) (31), at 600 nm (32). Log phase procyclic cells were harvested and washed twice with phosphate-buffered saline, pH 7.9, before being resuspended in 500 μl of TSE buffer (25 mM Tris-HCl, pH 8.0, 1 mm EDTA, 10% (v/v) glycerol) and by sonication for PRODH activity (33, 34). The INT reaction mixture contained variable proline concentrations, 10 μl of 0.1% ethylene glycol, 0.4% (v/v) Tween 20; 0.16 μM Tris-HCl, pH 8.5, 0.04 mg of gelatin, and 0.5 mM INT. The DCPIP reaction mixture contained 11 mM MOPS, 11 mM MgCl₂, 11% (v/v) glycerol, 0.28 mM phenazine methosulfate, and 56 μM of DCPIP, pH 7.5. Variable proline concentrations were added to 900–950 μl of the stock reaction mixture containing 1 μl of enzyme (1–5 μl).

Activity was monitored spectrophotometrically in cuvettes with a 1-cm light path at 600 nm.

RNA Interference (RNAi)—A 606-bp, XbaI-flanked, fragment of the PRODH gene was amplified by PCR from T. brucei cDNA using specific primers each containing an XbaI restriction site (5′-ATTTCGCAACCCCTCACTGCTTATGTTTATTG3′). The amplification was performed on the Biocad 700E work station using Ni2+ chelate chromatography (Novagen) with the elution buffer recommended by the manufacturer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 500 mM imidazole). Antiserum was raised in rabbits (Diagnostics Scotland), with an initial injection of 0.4 ml of protein solution at 1 mg ml−1 followed by three more 0.2-ml injections at 1-month intervals. Antibodies were purified using an AminoLink® ( Pierce) kit carrying immobilized purified PRODH on a solid support. Purified antibodies were aliquoted and stored at −20 °C until use. Western blotting of SDS-PAGE separated proteins on nitrocellulose membranes involved blocking for 3 h at room temperature with 1× TBS (0.02 mM Tris-HCl, 13.7 mM NaCl) containing 5% (v/v) powdered milk and 0.2% (v/v) Tween 20. The primary antibody, anti-PRODH (rabbit polyclonal antibody diluted 1:2000) or specific PRODH antibody (diluted 1:500 to 1:5000), was made up in 1× TBS, 1% (v/v) powdered milk, and 0.1% (v/v) Tween 20, before being added to the membrane and shaken overnight at 4 °C. The SuperSignal chemiluminescent substrate (Pierce) protocol with 1:2000 diluted anti-mouse IgG conjugated to peroxidase or with 1:500 to 1:5000 anti-rabbit horseradish peroxidase-coupled IgG (Sigma) allowed the detection of the proteins according to manufacturer’s specifications.

Production of Anti-PRODH Antibodies and Protein Analysis—Redemembrant PRODH lacking the N-terminal 72 amino acids that included the mitochondrial targeting sequence and a putative transmembrane domain (preliminary experiments showed that these domains precluded soluble expression) was created after amplification using primers TpPRODH2 (5′-AAACATATGACGGTGCT-GAGCAATTATTTT) and Tpvre (5′-CCCGACTTACCATCAACAGG-GCG). The product was ultimately cloned into pET21a+ and expressed in Escherichia coli strain BL21(DE3). Protein was produced in soluble form after expression at 16 °C in the presence of 1 mM isopropyl β-D-thiogalactopyranoside. The purification was performed on the Biocad 700E work station using Ni2+ chelate chromatography (Novagen) with the elution buffer recommended by the manufacturer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 500 mM imidazole). Antiserum was raised in rabbits (Diagnostics Scotland), with an initial injection of 0.4 ml of protein solution at 1 mg ml−1 followed by three more 0.2-ml injections at 1-month intervals. Antibodies were purified using an AminoLink® ( Pierce) kit carrying immobilized purified PRODH on a solid support. Purified antibodies were aliquoted and stored at −20 °C until use. Western blotting of SDS-PAGE separated proteins on nitrocellulose membranes involved blocking for 3 h at room temperature with 1× TBS (0.02 mM Tris-HCl, 13.7 mM NaCl) containing 5% (v/v) powdered milk and 0.2% (v/v) Tween 20. The primary antibody, anti-PRODH (rabbit polyclonal antibody diluted 1:2000) or specific PRODH antibody (diluted 1:500 to 1:5000), was made up in 1× TBS, 1% (v/v) powdered milk, and 0.1% (v/v) Tween 20, before being added to the membrane and shaken overnight at 4 °C. The SuperSignal chemiluminescent substrate (Pierce) protocol with 1:2000 diluted anti-mouse IgG conjugated to peroxidase or with 1:500 to 1:5000 anti-rabbit horseradish peroxidase-coupled IgG (Sigma) allowed the detection of the proteins according to manufacturer’s specifications.
parasite ml⁻¹. Radiolabeled L-[2,3,4,5-³H]proline (112 Ci mmol⁻¹) (PerkinElmer Life Sciences) was used in uptake assays. Uptake involved the oil stop transport assay as described (37, 38) with 100 µl of the test solution cushioned above 90 µl of oil mixture (di-n-butylphthalate and mineral oil at a ratio of 7:1). 100 µl of cells (at 10⁶ cell ml⁻¹) were added to each tube and incubated for times ranging between 3 s and 3 h depending on the experiment. The transport assays were terminated by centrifugation (12,000 x g, 1 min at room temperature). Samples were flash-frozen in liquid nitrogen before removing the pellets using a tube cutter and transferring them to scintillation vials. Samples were flash-frozen in liquid nitrogen before removing the pellets using a tube cutter and transferring them to scintillation vials.

RESULTS

Increase of Proline Consumption in T. brucei Cultivated in Glucose-depleted Medium—The SDM79 semidefined medium has become the growth medium of choice for procyclic form T. brucei (16). This insect stage parasite, grown in SDM79 or equivalent media, primarily consumes glucose as a carbon source and also depletes medium of threonine and to a lesser extent proline, glutamine, and pyruvate (15, 19) at significant rates. We previously estimated that the rate of glucose consumption of the EATRO1125 strain grown in the SDM79 medium is about 3-fold higher than the rate of proline consumption, during mid-log phase growth (19). To determine whether the rate of consumption of other potential carbon sources is influenced by the absence of glucose, the EATRO1125 strain was adapted to the glucose-depleted medium SDM80 (0.15 mM glucose), a derivative of SDM79 (6 mM glucose). The 40-fold reduction in glucose concentration does not affect the growth rate of this procyclic cell line nor that of the T. brucei 427 strain (with doubling time being the same in SDM80, SDM80 containing 6 mM glucose, and SDM79 media (Fig. 2, top panels)).

For the EATRO1125 line, the quantity of each of the 20 amino acids, and pyruvate, present in the growth medium, was determined for cells grown in the glucose-depleted (0.15 mM) and glucose-rich (6 mM) SDM80 media. For some amino acids, the rate of consumption (threonine, pyruvate, and glutamine) or production (glycine, alanine, and glutamate) was not different regardless of the medium used. The concentrations of other amino acids were not significantly altered in either medium. However, the rate of proline consumption increased severalfold in the glucose-depleted medium (Fig. 2). To quantify the relative proline consumption increase during the mid-log phase, both the EATRO1125 and 427 strains were incubated at a higher cell density (5 x 10⁶), and the proline concentration in the growth medium was determined periodically over a period of 2 days (Fig. 3). The rate of proline consumption is ~2-fold higher for the 427 cell line than for the EATRO1125 cell line in the presence (0.15 versus 0.31 µmol h⁻¹ mg protein⁻¹) or the absence (1.8 versus 0.93 µmol h⁻¹ mg protein⁻¹) of glucose. These differences may be related to the higher growth rate observed for the 427 strain (doubling time: 10.5 versus 13.8 h). However, the rate of proline consumption in the glucose-depleted medium is ~6-fold increased in both EATRO1125 (6.3-fold) and 427 (5.7-fold) strains.

We also attempted to grow the 427 cell line in medium lacking both glucose and proline but containing each of the other 19 commonly used L-amino acids at 10 mM. Proline, but no other amino acid, could support robust growth in glucose-depleted conditions (Fig. 4). This included L-glutamate, a direct product of the proline degradation pathway and glutamine, which is also converted into glutamate. Moreover, L-threonine could not support growth despite the fact that this amino acid is consumed in great quantities by procyclic T. brucei. In other experiments (not shown) proline was also included at low con-

FIG. 2. Substrate utilization and metabolite excretion by T. brucei EATRO1125 procyclic cells grown in the presence (A) or the absence (B) of glucose. Aliquots of the growth medium (SDM80 with or without glucose), inoculated with 10⁶ EATRO1125 procyclic cells ml⁻¹, were collected twice a day over 4 days. Cell number (top of the figure) and the concentration of D-glucose, pyruvate, and all the amino acids were determined. Only the values obtained for L-alanine (Ala), L-glutamate (Glu), L-glutamine (Gln), L-glycine (Gly), L-proline (Pro), and L-Threonine (Thr) are shown in the lower part of the figure. There was no significative measurable change in the concentrations of all other amino acids present in the medium. The arrowheads indicate when D-glucose, pyruvate, L-glutamine, and L-threonine were no longer detectable in the medium.
centration (0.1 M) to ensure that non-energy requirements of this amino acid (e.g. protein biosynthesis) were provided. The presence of proline did not affect the outcome.

Oxidative Phosphorylation Is Essential for Growth in Glucose-depleted Medium—We previously observed that in glucose-rich medium, pyruvate kinase, which produces ATP in the cytosol by substrate level phosphorylation, is essential for procyclic trypanosomes. In contrast, oligomycin (the most specific inhibitor of the mitochondrial F0/F1-ATP synthase) does not affect the steady state amount of intracellular ATP and only moderately affects parasite growth (19). We proposed that production of ATP by substrate level phosphorylation is essential, while ATP generation by the F0/F1-ATP synthase (oxidative phosphorylation) is available but not essential (19) for procyclic cells grown in glucose-rich medium. In the absence of glucose, the overall carbon source consumption decreases significantly, although the relative rate of proline consumption increases. We determined the oligomycin concentration required to kill all of the cells (LD100) grown in glucose-rich or glucose-depleted medium (Fig. 5). At day 3 of incubation with oligomycin, the EATRO1125 and 427 strains are 2000 and 5000 times more sensitive to the metabolic effector, respectively, when grown in glucose-depleted medium as compared with glucose-rich medium. This indicates that production of ATP by oxidative phosphorylation is essential for procyclic cell viability when catabolizing substrates other than those that can pass through the glycolytic pathway.

Proline Uptake and Metabolism by Procyclic T. brucei Grown in Glucose-depleted Medium—To study the reason for the increase in the rate of proline consumption in the glucose-depleted medium, we measured the activity of the two first steps of proline metabolism (i.e. proline transport and proline dehydrogenase) in procyclic trypanosomes. Proline uptake in procyclic trypanosomes (strain STIB 366) grown in glucose-rich medium has been previously shown to be carrier-mediated with an apparent $K_m$ for proline of 19 μM (39). Here we show that the apparent $K_m$ of proline uptake was similar for the 427 strain grown in glucose-rich (21 ± 2.9 μM) and glucose-depleted (18 ± 0.0042 μM) media (Fig. 6A). Using a 30 ± time point, the apparent maximum capacity of the proline transporter ($V_{max}$) is 2.6 times higher in cells grown in the absence of glucose (1.8 ±
0.13 nmol·min$^{-1}$·10$^7$ parasites$^{-1}$ versus 0.7 ± 0.06 nmol·min$^{-1}$·10$^7$ parasites$^{-1}$ (Fig. 6A).

The specific activity of PRODH, which catalyzes the oxidation of proline into Δ-pyrroline-5-carboxylate, which is then hydrolyzed non-enzymatically to yield glutamate γ-semialdehyde was measured in the 427 strain grown in both conditions (Fig. 6B). PRODH activity is 2-fold higher in cells grown in glucose-depleted medium (0.054 ± 0.004 μmol·min$^{-1}$·mg protein$^{-1}$) as compared with cells grown in glucose-rich conditions (0.027 ± 0.004 μmol·min$^{-1}$·mg protein$^{-1}$).

To test whether repression of proline metabolism is a direct result of glucose availability, or a secondary effect (for example due to abundant ATP synthesis in the presence of glucose or other metabolites), glucose was replaced by its analogue 2-deoxy-D-glucose. The analogue does not inhibit growth of wild-type cells in glucose-rich medium. However, the 427 procyclc cells ceased to grow when 2-deoxy-D-glucose was added to proline-rich, glucose-free, medium (data not shown). This may indicate that 2-deoxy-D-glucose stimulates a down-regulation in proline metabolism in a fashion similar to glucose itself despite its incapacity to yield ATP or to be metabolized beyond 2-deoxy-D-glucose 6-phosphate.

The T. brucei PRODH Gene—PRODH genes from other sources were used to query the T. brucei genome data base for orthologs. A single gene (Tb07.8P12.290) located on chromosome VII was found. Analysis of reverse transcriptase PCR products using an internal oligonucleotide and nested primers (data not shown). The predicted open reading frame is 1668 nucleotides encoding a predicted protein of 556 amino acids similar to other PRODH (predicted molecular mass of 63.8 kDa). For instance, the Tb07.8P12.290 gene product is 30% identical and 53% similar to the E. coli proline dehydrogenase (which shares 32% similarity, 23% identity with the T. brucei enzyme) has been crystallized and key residues involved in substrate and cofactor (FAD) binding are known. In the E. coli structure, Lys$^{329}$, Arg$^{555}$, Arg$^{556}$, Asp$^{770}$, Tyr$^{540}$, and Leu$^{513}$ have all been implicated in substrate binding. All are also present in T. brucei except Leu$^{513}$ which is replaced with a related residue, valine. Arg$^{431}$, which plays a key role in FAD binding in the E. coli enzyme is also conserved, indicating that the enzymatic mechanism is likely to be conserved between E. coli and trypanosomes. In prokaryotes, the PRODH gene is fused to that encoding pyrroline 5-carboxylate dehydrogenase, the enzyme that follows PRODH in the proline degradation pathway. The two genes are separated in eukaryotes, including T. brucei (a pyrroline 5-carboxylate dehydrogenase ortholog is present on T. brucei chromosome III). In all eukaryotes analyzed to date, PRODH localizes to the inner membrane of the mitochondrion (40, 41). The T. brucei enzyme also has a predicted N-terminal mitochondrial targeting sequence, followed by a putative transmembrane spanning domain that is predicted to be involved in associating this enzyme to the inner membrane of the mitochondrion in T. brucei (40, 41).

Loss of PRODH Expression Is Conditionally Lethal to Procyclic T. brucei—RNAi was used to learn more about the role of PRODH in the metabolism of procyclic T. brucei. Parasites transfected with a construct carrying 606 nucleotides of the PRODH gene between two T7 promoters in the p2T7 vector (35) were induced to produce double stranded RNA copies of that insert by tetracycline. The apodh F2 clone was selected for further analyses. Within 4 h of induction the PRODH RNA transcript was substantially depleted (and no transcript could be detected by 24 h) (Fig. 7A). Moreover, Western blotting using antibodies to the purified overexpressed enzyme revealed the protein to be absent from the tetracycline-induced apodh F2 cell line (Fig. 7B) and PRODH activity is not detectable in the tetracycline induced cells within 48 h (Fig. 7C). This confirms that the PRODH gene indeed encodes PRODH in T. brucei. In the glucose-rich medium (with or without proline), the PRODH-deficient cells grew at a rate and to a density similar to uninduced cells (Fig. 7D). Neither the induced nor non-induced cells can grow in the absence of glucose and proline, as observed for the wild-type 427 cell line. However, in the glucose-depleted medium containing proline, the PRODH-deficient cell line ceased to grow, while the uninduced cells were not affected. To determine the effect of an absence of proline dehydrogenase on proline uptake activity, the uptake of proline was also measured in PRODH-deficient cells. The affinity for proline was similar in the 427 (29-13 cell line) with or without PRODH when grown in glucose-rich or glucose-depleted medium (K$m = 11 ± 0.003 \mu M$ for uninduced versus K$m = 12 ± 0.004 \mu M$ for induced cells). The apparent $V_{max}$ values, however, differed (0.75 ± 0.06 nmol·min$^{-1}$·10$^7$ cells$^{-1}$ when PRODH was present versus 0.41 ± 0.04 nmol·min$^{-1}$·10$^7$ cells$^{-1}$ when PRODH was absent) (Fig. 8A). The decrease in apparent $V_{max}$ could be explained either as a result of measured uptake at 30 s representing combined transport and proline dehydrogenase linked metabolism, or it could represent an independent decrease in transport rate. To attempt to resolve this issue we also measured uptake over a 3-s time period, the shortest time we could reliably use with the oil stop technique (Fig. 8B). If measured uptake represents a combined measurement of uptake and metabolism, it might be expected that the apparent maximal rate of uptake would converge in wild-type and PRODH-deficient cells. The apparent $V_{max}$ values measured at 3 s rather than 30 s were faster (1.5 nmol·min$^{-1}$·10$^7$ cells$^{-1}$ for the PRODH mutant and 2.7 nmol·min$^{-1}$·10$^7$ cells$^{-1}$ for wild type), thus demonstrating that at 30 s the linear phase of transport is already over. However, the difference between wild-type and PRODH-deficient cells with respect to apparent
maximal rate of uptake was retained. This could indicate that proline transport activity declines in response to a decline in proline dehydrogenase activity. It may therefore be concluded that both proline transport and its subsequent metabolism are down-regulated when cells are exposed to high concentrations of glucose.

**DISCUSSION**

The *T. brucei* bloodstream stumpy-form, ingested by the tsetse fly during the blood meal, differentiates in the midgut of the insect vector into the procyclic form. After differentiation into the procyclic form, the parasite resides in an environment that probably lacks glucose, as this is consumed rapidly to virtual absence within 15 min in the tsetse midgut subsequent to its taking the blood meal (9). The digestive contents of the gut are considered to be rich in amino acids, as is the tsetse hemolymph, which provides proline, the principal energy source used during flight of the tsetse fly (11). Procyclic trypanosomes have been adapted to *in vitro* axenic culture, usually in glucose-rich media exemplified by the commonly...
used SDM79 medium, which contains this sugar at 6 mM. Most studies into procyclic metabolism have been conducted under these glucose-rich conditions.

When grown in glucose-rich medium, several strains of procyclic trypanosomes consume glucose, threonine, proline, and glutamine in relatively high abundance (Refs. 15 and 19 and this report). Interestingly, there are strain differences in substrate utilization between different isolates. For example, the EATRO1125 strain (Ref. 19 and this report) consumes abundant pyruvate but not glutamate, while the S42 strain (15) consumes glutamate but not pyruvate. Nevertheless, it appears that all of the procyclic strains analyzed so far consume glucose and threonine and, to a lesser extent, proline and glutamine.

Here, using both the EATRO1125 and 427 procyclic strains, we show for the first time that in medium containing a low glucose concentration (0.15 mM), trypanosomes increase their rate of proline consumption (6-fold) as compared with growth in glucose-rich medium (6 mM). The rate of consumption of the other carbon sources, however, is not significantly affected. Glucose thus appears to exert a negative control on proline metabolism (5 mM proline), which implies that glucose is the preferred carbon source, in conditions of relative glucose abundance. These data contrast with previous reports that suggested a preference for proline over glucose as a carbon and energy source (12–14, 42). For instance, ter Kuile (42) showed that in the presence of a large excess of proline (50 mM) over glucose (2.5 mM), glucose consumption was 2–4-fold lower than in cells grown in medium containing 5 mM glucose and 0.5 mM proline. However, none of these previous experiments were performed in conditions that reflect the normal, low glucose, environment of the tsetse fly midgut between blood meals. The raison d’être of the negative regulation exerted by glucose on proline metabolism is not clear. One may consider that immediately following the blood meal glucose is the main carbon source and proline metabolism is down-regulated, then, as glucose availability diminishes, negative regulation of proline metabolism is relieved, although if, as suggested, glucose is depleted to negligible levels within 15 min of the blood meal (9), this would seem unlikely.

Amino acids other than proline failed to sustain growth of *T. brucei* in SDM80 medium depleted in glucose, even when added to 10 mM. Threonine is consumed in relatively high quantities by procyclic trypanosomes but it failed to support growth indicating that its principal use, as suggested previously (43), is in provision of 2-carbon units for lipid biosynthesis. It appears that this amino acid is metabolized to glycine and acetate, which are secreted in high abundance, suggesting a profligate use of threonine by procyclic trypanosomes. Neither glutamate nor glutamine could support growth, despite the fact that glutamate is an intermediate in the proline degradation pathway. Failure of these amino acids to support growth could relate to issues of uptake into the cell or the mitochondrion.

To learn more about the points within the proline catabolic pathway that are affected by glucose, we studied both proline uptake and PRODH activity. PRODH activity is diminished in cells grown in the presence of abundant glucose when compared with cells where glucose is limiting. Moreover, proline uptake is also diminished in cells grown under conditions of abundant glucose. It seems that the decrease in proline transport is a response to the down-regulation of proline metabolism, given that the rate of transport appears to be diminished in PRODH deficient cells. Down-regulation of PRODH expression appears not to be mediated at the transcriptional level nor at the level of RNA stability, since Northern blots indicate no decrease in levels of the steady state transcript (data not shown).

Interestingly, the carbon source switch is associated with important metabolic changes in procyclic trypanosomes, as exemplified by the increased sensitivity to the most specific known inhibitor of the mitochondrial F<sub>0</sub>/F<sub>1</sub>-ATP synthase (oligomycin) when parasites are grown under glucose-depleted conditions. In the procyclic trypanosomes, the F<sub>0</sub>/F<sub>1</sub>-ATP synthase exploits the proton gradient generated by the respiratory chain to generate ATP by oxidative phosphorylation (44). Oxidative phosphorylation was long considered to be the main source of ATP in procyclic cells (5, 6, 44). However, the essential role of the F<sub>0</sub>/F<sub>1</sub>-ATP synthase in energy production, under glucose-rich conditions, has recently been questioned (19). Here we show that cells grown in the absence of glucose are killed by 10 ng ml<sup>−1</sup> oligomycin (corresponding to 0.2 μg mg<sup>−1</sup> protein), which represents only 10% of the dose required to fully inhibit the mitochondrial F<sub>0</sub>/F<sub>1</sub>-ATP synthase (19, 45). This suggests that a partial inhibition of the F<sub>0</sub>/F<sub>1</sub>-ATP synthase is sufficient to kill the procyclic cells grown in the absence of glucose or that the drug accumulates to internal concentrations exceeding those outside the cell. However, the same cell line grown under glucose-rich conditions is 2000–5000 times less sensitive to oligomycin (200–500 times the dose required to inhibit the F<sub>0</sub>/F<sub>1</sub>-ATP synthase). These data support a view that, in the presence of glucose, procyclic cells are not dependent on oxidative phosphorylation for ATP production, but in the absence of glucose, when cells switch to proline metabolism, oxidative phosphorylation becomes essential.

Proline dehydrogenase is directly associated with the inner membrane of the mitochondrion in eukaryotes, and the presence of a mitochondrial targeting sequence and transmembrane domain indicate that PRODH in trypanosomes is similarly localized. Electrons are transferred directly from proline to the electron transport chain via the FAD cofactor bound to proline dehydrogenase (31). In *T. brucei*, in addition to the contribution of these electrons to the respiratory chain, additional energy may be derived from the generation of NADH created by each of the next three steps in the pathway: pyrroline-5-carboxylate dehydrogenase, glutamate dehydrogenase, and 2-ketoglutarate dehydrogenase (Fig. 1). Finally the succinyl-CoA synthetase will generate additional ATP as succinate is generated as the end product of proline metabolism, thus indicating why proline can be used as a relatively rich energy source in these cells.

It is not immediately obvious what role the regulation of proline metabolism may play in the physiology of the parasites; however, it is of note that a number of other processes appear to be sensitive to glucose availability in procyclic *T. brucei*. For example, expression of the main surface protein-encoding *PARP* genes (GPEET or EP varieties) appears to be regulated in response to glucose levels (46, 47). Whether there is a general glucose-response pathway in procyclic cells is a topic of great interest. *PARP* gene expression also appears to respond to levels of mitochondrial metabolic intermediates (48).

In the case of procyclic metabolism, by contrast, the response might be directly related to glucose, since the presence of the non-metabolizable glucose analogue, 2-deoxy-o-glucose, alone is sufficient to block growth of parasites using proline as an energy source. It is possible that 2-deoxy-o-glucose affects cells in a way independent of a down-regulation of proline metabolism when glucose is absent, but since cells do not grow under these conditions alternative approaches are required to assess this.

In summary it appears that proline is a principal source of energy for procyclic trypanosomes when glucose abundance is limiting, as found in the tsetse fly. Other amino acids do not
appear to be able to replace proline as an energy source. However, when glucose is abundant, the cells preferentially use this carbohydrate and down-regulate proline metabolism in a manner which involves diminished activity of both proline transport and PRODH. It will be of interest to determine whether the response of procyclies to glucose with regard to proline metabolism is linked to the response in expression of particular PARP surface membrane protein isoforms and/or is related to processes leading to the differentiation of procyclies into epimastigotes.

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Proline Metabolism in Procyclic Trypanosoma brucei Is Down-regulated in the Presence of Glucose
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