

Glucose-induced Remodeling of Intermediary and Energy Metabolism in Procyclic *Trypanosoma brucei*^{*[5]}

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The procyclic form of *Trypanosoma brucei* is a parasitic protozoan that normally dwells in the midgut of its insect vector. *In vitro*, this parasite prefers D-glucose to L-proline as a carbon source, although this amino acid is the main carbon source available in its natural habitat. Here, we investigated how L-proline is metabolized in glucose-rich and glucose-depleted conditions. Analysis of the excreted end products of ¹³C-enriched L-proline metabolism showed that the amino acid is converted into succinate or L-alanine depending on the presence or absence of D-glucose, respectively. The fact that the pathway of L-proline metabolism was truncated in glucose-rich conditions was confirmed by the analysis of 13 separate RNA interference-harboring or knock-out cell lines affecting different steps of this pathway. For instance, RNA interference studies revealed the loss of succinate dehydrogenase activity to be conditionally lethal only in the absence of D-glucose, confirming that in glucose-depleted conditions, L-proline needs to be converted beyond succinate. In addition, depletion of the F₀/F₁-ATP synthase activity by RNA interference led to cell death in glucose-depleted medium, but not in glucose-rich medium. This implies that, in the presence of D-glucose, the importance of the F₀/F₁-ATP synthase is diminished and ATP is produced by substrate level phosphorylation. We conclude that trypanosomes develop an elaborate adaptation of their energy production pathways in response to carbon source availability.

Trypanosomatids are parasitic protozoa, among which several species cause serious diseases in humans such as sleeping sickness (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and leishmaniasis (*Leishmania* spp.). These pathogenic trypanosomatids have developed a digenetic lifestyle with one

or several vertebrate hosts (including humans) and a hematophagous insect vector that allows their transmission between vertebrate hosts. Recently, the genome sequencing projects of *T. brucei* (TREU927 strain) (1), *T. cruzi* (CL Brener strain) (2), and *Leishmania major* (Friedlin strain) (3) have been completed, providing wonderful tools to determine their metabolic complexities (1).

Trypanosomatids depend on the carbon sources present in their hosts for their energy metabolism (4). For example, the trypomastigote forms of *T. brucei* and *T. cruzi* (bloodstream forms) use D-glucose, which is abundant in the fluids of their vertebrate host(s) (5, 6). In contrast, the insect vectors obtain their energy from L-proline and/or L-glutamine, the prominent constituent of their hemolymph and tissue fluids (7). Consequently, the insect stages of *T. brucei* and *T. cruzi* rely on amino acid catabolism, with a preference for L-proline. However, these parasites prefer D-glucose when grown in medium rich in this sugar. Because glucose-rich media are routinely used to grow these parasites, D-glucose metabolism has received the most attention, and relatively little is known about their amino acid metabolism. Recent advances in understanding about trypanosomatid catabolism have focused on procyclic *T. brucei*, which has significant overlap with other species regarding its metabolism, but for which RNA interference (RNAi)² has been extensively developed. RNAi is not functional in *T. cruzi* or in most *Leishmania* subspecies (8), although it appears that *Leishmania braziliensis* does have the machinery required for RNAi (9).

Procyclic trypanosomes grown in rich media primarily convert D-glucose into succinate and acetate, with smaller amounts of lactate and CO₂ (10, 11). Succinate represents ~70% of the excreted end products of D-glucose metabolism. It is produced in the glycosomes (peroxisome-like organelles specialized in D-glucose metabolism in trypanosomatids) and the mitochondrion by two NADH-dependent fumarate reductase isoforms, FRDg and FRDm1 (mitochondrial NADH-dependent fumarate reductase), respectively (10, 12, 13). Acetyl-CoA produced in the mitochondrion is not metabolized to CO₂ through the tricarboxylic acid cycle (11), but is converted into acetate by the

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² The abbreviations used are: RNAi, RNA interference; FRDg, glycosomal fumarate reductase; SDH, succinate dehydrogenase; PDH, pyruvate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; MEc, cytosolic malic enzyme; MEm, mitochondrial malic enzyme; ATPε-F1β, F₁ complex β-subunit; PBS, phosphate-buffered saline.

mitochondrial acetate:succinate CoA-transferase and another as yet unknown enzymatic activity (14, 15).

Procyclic forms of several *T. brucei* strains were successfully adapted to glucose-depleted medium, with no significant effect on growth rate (16–18). The rate of L-proline consumption in these growth conditions is increased by ~6-fold (18). It has been proposed that the procyclic form of *T. brucei*, as well as other trypanosomatids, adapts its energy metabolism to available carbon sources (4); however, this view has been recently questioned (19, 20). Here, we illustrate the metabolic flexibility of procyclic trypanosomes by comparing their L-proline metabolism when grown in glucose-rich and glucose-depleted media. In glucose-rich conditions, the L-proline degradation pathway is truncated, and complexes II and IV of the respiratory chain and the mitochondrial F_0/F_1 -ATP synthase become of greatly reduced significance to the energy pathways of the cell. We conclude that D-glucose availability leads to down-regulation of the proline degradation pathway, along with proton flow through the mitochondrial F_0/F_1 -ATP synthase and electron flow through the respiratory chain (in addition to negative regulation of the rate of L-proline consumption).

EXPERIMENTAL PROCEDURES

Trypanosome and Cell Cultures—The procyclic form of *T. brucei* EATRO1125 was cultured at 27 °C in SDM79 medium containing 10% (v/v) heat-inactivated fetal calf serum and 3.5 mg/ml hemin (21) or in a glucose-depleted medium derived from SDM79, called SDM80 (18). SDM80 medium is supplemented with 9% (v/v) heat-inactivated fetal calf serum dialyzed by ultrafiltration against 0.15 M NaCl (molecular mass cutoff of 10,000 Da; Sigma F0392; D-glucose concentration of 1 mM) and 1% (v/v) heat-inactivated fetal calf serum (D-glucose concentration of 5 mM). The D-glucose concentration in SDM80 medium is 0.15 mM compared with 6 mM in SDM79 medium and 6.15 mM in SDM80 medium supplemented with D-glucose (SDM80glu).

Inhibition of Gene Expression by RNAi—The inhibition by RNAi of gene expression in the procyclic forms (22) was performed by expression of stem-loop “sense/antisense” RNA molecules of the targeted sequences (23) introduced into the pLew100 or pLew79 expression vector (kindly provided by E. Wirtz and G. Cross) (24) or the p2T7^{Ti}-177 vector (kindly provided by B. Wickstead and K. Gull) (25) as described previously (10, 12, 13, 15, 23, 26). These three vectors contain the phleomycin resistance gene. Construction of the pLew-ASCT-SAS, pLew-FRDm1-SAS, pLew-FRDg-SAS, pLew-FRDg/m1-SAS, pLew-FHm-SAS, pLew-FHc-SAS, and p2T7^{Ti}-177-FHc/m-SAS plasmids, used to produce the ^{RNAi}ASCT-hp4, ^{RNAi}FRDm1-G7, ^{RNAi}FRDg-C2, ^{RNAi}FRDg/m1-B5, ^{RNAi}FHm-A1, ^{RNAi}FHc-A5 and ^{RNAi}FHc/m-F10 cell lines, respectively, has been described (12, 13, 15) (the ^{RNAi}XXX cell lines were previously called ΔXXX).

The pLew-SDH-SAS construct targets a fragment (bp 12–452) of the succinate dehydrogenase (SDH) gene (*Tb09.160.4380*). Briefly, a PCR-amplified 524-bp fragment containing the antisense SDH sequence (440 bp of targeted sequence plus 96 bp used as a spacer to form the loop between the annealing sense and antisense sequences, respectively) with

restriction sites added to the primers was inserted into the HindIII and BamHI restriction sites of the pLew100 plasmid. Then, a PCR-amplified 474-bp fragment containing the sense SDH sequence was inserted upstream of the antisense sequence using HindIII and XhoI restriction sites (XhoI was introduced at the 3'-extremity of the antisense PCR fragment). The resulting plasmid (pLew-SDH-SAS) contains a sense and antisense version of the targeted gene fragment, separated by a 96-bp fragment, under the control of the *PARP* promoter linked to a prokaryotic tetracycline operator.

The pLew-PDH.E2-SAS plasmid (designed to inhibit, by RNAi, the expression of the E2 subunit of the pyruvate dehydrogenase (PDH) complex) was generated in the pLew79 vector with the same strategy described above, employing the same restriction sites. The targeting cassette is composed of a sense and antisense version of the *Tb10.6k15.3080* gene (bp 69–632), separated by an 84-bp fragment.

To target inhibition of expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene (*Tb927.2.4210*), the 3'-end of the coding sequence and the 3'-untranslated region were selected. The targeted sequences correspond to the last 130 bp (bp 1448–1578 of the PEPCK gene) of the coding sequence followed by 197 bp of the 3'-untranslated region. The sense and antisense fragments, separated by 40 bp, were cloned into the pLew100 vector.

To simultaneously target both malic enzyme genes, which encode the cytosolic and mitochondrial isoforms (MEc (*Tb11.02.3120*) and MEm (*Tb11.02.3130*), respectively), we selected a fragment from the MEc gene (bp 427–824) that showed the highest homology to the MEm gene (80% identity). The sense and antisense fragments, separated by 26 bp, were cloned in the pLew100 vector. To inhibit expression of the mitochondrial F_0/F_1 -ATP synthase, the sense and antisense versions of a fragment (bp 471–934) of the β -subunit gene of the F_1 complex (ATP ϵ -F1 β ; *Tb927.3.1380*), separated by a 58-bp fragment, were cloned into the BamHI and HindIII restriction sites of the p2T7^{Ti}-177 vector.

Trypanosome Transfection, Adaptation to SDM80 Medium, and RNAi Induction—The EATRO1125 procyclic form cell line (EATRO1125.T7T), constitutively expressing the T7 RNA polymerase gene and the tetracycline repressor under the control of a T7 RNA polymerase promoter for tetracycline-inducible expression (23), was transformed with each of the plasmids. Trypanosome transfection and selection of phleomycin-resistant clones were performed as reported previously (27). Briefly, all of the RNAi-harboring cell lines were selected in glucose-rich SDM79 medium containing hygromycin B (25 μ g/ml), neomycin (10 μ g/ml), and phleomycin (5 μ g/ml). Aliquots of each were frozen in liquid nitrogen to provide stocks of each line that had not been cultivated long term in medium. The selected cell lines were then adapted to glucose-rich SDM80glu medium and glucose-depleted SDM80 medium containing the same concentration of the three antibiotics. The cell lines were first transferred to SDM80glu medium and maintained for at least 2 weeks before adaptation to SDM80 medium. All analyzed cell lines were maintained for 2 weeks in SDM80 or SDM80glu medium before addition of 1 μ g/ml tetracycline for RNAi induction.

Enzyme Assays—Sonicated (5 s at 4 °C) crude extracts of trypanosomes resuspended in cold hypotonic buffer (10 mM potassium phosphate, pH 7.8) were tested for pyruvate kinase (EC 2.7.1.40) (28), fumarase (EC 4.2.1.2) (13), FRD (EC 1.3.1.6) (10), PDH (EC 1.2.2.2) (29), ME (EC 1.1.1.40) (30), and SDH (EC 1.3.99.1) (31) activities as described.

Western Blot Analyses—Total protein extracts of the procyclic form of *T. brucei* (2×10^6 cells) were separated by SDS-PAGE (10%) and immunoblotted on Immobilon-P filters (Millipore) (32). Immunodetection was performed as described (32, 33) using, as primary antibodies, the rabbit antiserum against the F_1 moiety of the mitochondrial F_0/F_1 -ATP synthase isolated from *Crithidia fasciculata* (a gift from D. Speijer) (34) and the rabbit antiserum against glycerol-3-phosphate dehydrogenase (27, 35) diluted 1:100. Peroxidase-conjugated goat anti-mouse or anti-rabbit Ig (1:10,000 dilution) was used as a secondary antibody, and revelation was performed using ECLTM Western blotting detection reagents (Amersham Biosciences) as described by the manufacturer. For quantitative analyses, membranes or x-ray films were scanned, and protein bands were quantified using NIH Image software.

Determination of Sensitivity to Metabolic Drugs—EATRO1125 procyclic cells (2×10^6 cells/ml) were incubated in SDM80 medium either containing or lacking 6 mM D-glucose in the presence of decreasing quantities of rotenone (from 0.5 mM to 20 nM), salicylhydroxamic acid (from 10 mM to 1.5 μ M), potassium cyanide (from 10 mM to 80 nM), malonate (from 10 mM to 80 nM), and oligomycin (from 50 μ g/ml to 0.01 ng/ml). The assay was performed in 96-well microtiter plates with 100 μ l of cell suspension/well, and the cell viability was determined optically. The drug concentration required to kill all of the cells (100% lethal dose, LD₁₀₀) was determined 1, 2, 3, 4, and 5 days after drug addition.

D-Glucose and L-Proline Measurements—To determine the rate of D-glucose and L-proline consumption, cells (inoculated at $1\text{--}1.5 \times 10^7$ cells/ml) were grown in 10 ml of SDM80 (0.15 mM D-glucose) or SDM80glu (6.15 mM D-glucose) medium. Aliquots of each growth medium (500 μ l) were collected 0, 1, 6, 9, 10, 23, and 24 h after incubation at 27 °C. Cells were lysed by sonication in 500 μ l of 5 mM phosphate buffer, pH 7.5. The quantity of D-glucose present in the medium was determined using the Glucose GOD-PAP kit (Biolabo S.A.). L-Proline concentration was determined with a colorimetric assay as described previously (36) after deproteinization of the samples by perchloric acid treatment. At 0, 9, and 24 h, the cell density and cellular protein concentration were determined to estimate the amount of carbon source consumed per mg of cellular protein.

NMR Experiments— 6×10^8 *T. brucei* procyclic cells were collected by centrifugation at $1400 \times g$ for 10 min, washed once with phosphate-buffered saline (PBS), and incubated in 30 ml of incubation buffer (PBS supplemented with 24 mM NaHCO₃, pH 7.3). For the analysis of D-glucose metabolism, the cells were incubated for 6 h at 27 °C in incubation buffer containing 110 μ mol of D-[1-¹³C]glucose. For the analysis of L-proline metabolism, the cells were incubated in PBS, pH 7.3 (without NaHCO₃), containing 20 μ mol of L-[4-¹³C]proline and in the presence or absence of 100 μ mol of unenriched D-glucose or

100 μ mol of unenriched pyruvate. The D-glucose concentration in the medium was determined with the Glucose GOD-PAP kit. L-Proline concentration was determined with a calorimetric assay as described previously (36) after deproteinization of the samples by perchloric acid treatment. The integrity of the cells during the incubation was checked by microscopic observation. After centrifugation for 10 min at $1400 \times g$, the supernatant was lyophilized and redissolved in 485 μ l of D₂O, and 15 μ l 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 broadband probe head. Measurements were recorded at 25 °C under bilevel broadband gated proton decoupling with D₂O lock. Acquisition conditions were as follows: 90° flip angle, 22,150-Hz spectral width, 64K memory size, and 21.5-s total recycle time. Measurements were performed overnight with 2048 scans. Spectra were obtained after 1-Hz exponential line broadening. The specific ¹³C enrichment of lactate (C-3), acetate (C-2), and succinate (C-2 and C-3) was determined from ¹H-observed/¹³C-edited NMR (¹H/¹³C NMR) spectra acquired under ¹³C decoupling (37) according to a modification of the method described previously (38), wherein the detection of protons bound to ¹³C is based on the spin-spin coupling between directly bound ¹H and ¹³C nuclei ($J_{CH} = 127$ Hz). Two spectra were recorded from each sample; the first corresponded to protons bound to ¹²C and ¹³C carbons (spin-echo spectrum), and the second to protons bound to ¹³C carbons (¹³C-edited NMR spectrum). Flip angles for rectangular pulses were carefully calibrated on both radio frequency channels before each experiment. The relaxation delay was 8 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 ¹³C-edited NMR spectrum to its area in the standard spin-echo spectrum. The reproducibility and accuracy of the method were assessed using several mixtures of ¹³C-enriched amino acids and lactate with known fractional enrichments; the relative errors in the ¹³C enrichment determinations were <5%.

RESULTS

L-Proline Metabolism of Procyclic Trypanosomes in Glucose-depleted Medium—We used ¹³C NMR spectroscopy analysis to detect and quantify the metabolic end products excreted by the EATRO1125.T7T procyclic cell line grown in glucose-depleted medium and then incubated in PBS containing 2 mM L-[4-¹³C]proline. The incubation medium was analyzed by NMR spectroscopy. In these glucose-depleted conditions, the parasite excreted mainly ¹³C-enriched L-alanine (64.2%) and L-glutamate (28.2%) with small amounts of acetate (3.2%), β -hydroxybutyrate (3.1%), succinate (0.5%), L-aspartate (0.5%), and malate (0.3%) (Fig. 1A and Table 1).

We propose a model of L-proline metabolism in glucose-depleted conditions that tentatively accounts for the end products excreted by the procyclic trypanosomes (Fig. 2). It is based on current models in other organisms (39, 40) and the bioinformatics analysis of metabolic enzymes encoded by the *T. brucei* genome (Ref. 1; see also Table V in Ref. 19 for the *T. brucei* gene names encoding these enzymes). L-Proline is first converted in the mitochondrion into L-glutamate (Fig. 2, steps 1 and 2),

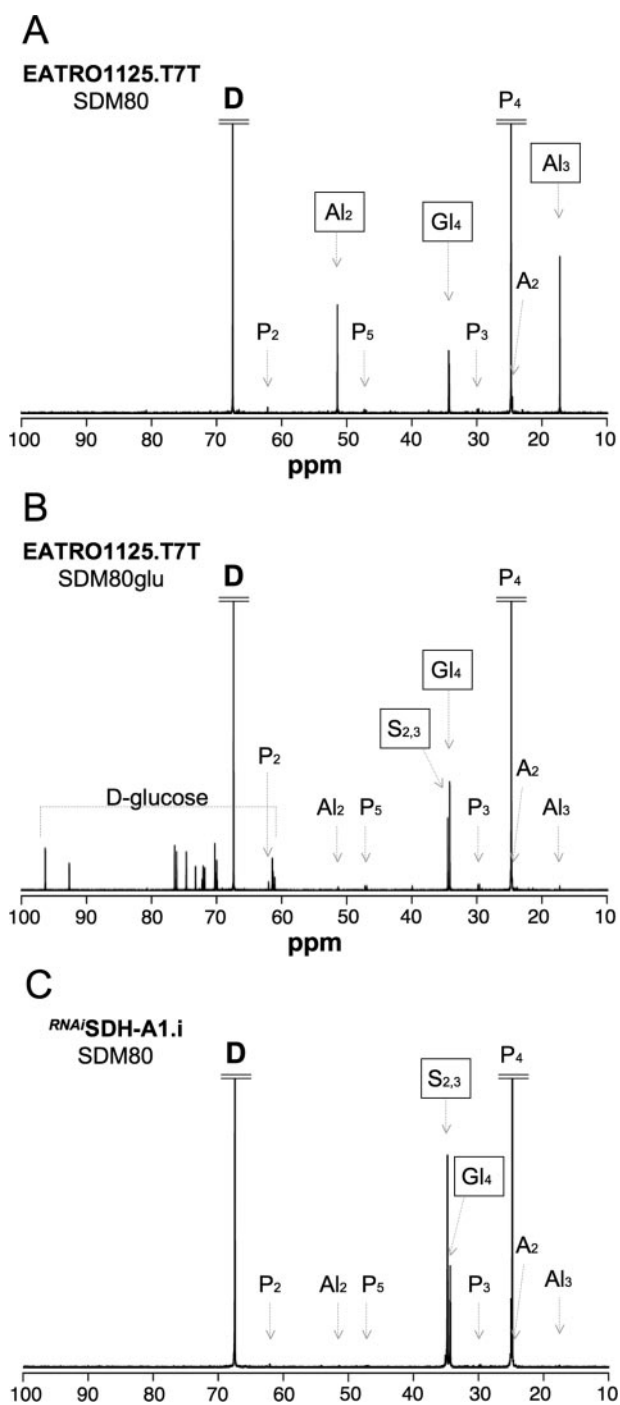


FIGURE 1. ^{13}C NMR spectra of metabolic end products excreted by procyclic cell lines incubated with L-[4- ^{13}C]proline. For these NMR analyses, the parental EATRO1125.T7T cell line grown in SDM80 glucose-depleted (A) or SDM80glu glucose-rich (B) medium and the tetracycline-induced RNAi-harboring cell line $^{\text{RNAi}}\text{SDH-A1.i}$ grown in SDM80 medium (C) were incubated with 2 mM L-[4- ^{13}C]proline in PBS with (B) or without (A and C) 3.5 mM D-glucose. The NMR spectra were obtained after addition of 15 μl dioxane. Each spectrum corresponds to one representative experiment from a set of three. The resonances were assigned as follows: A, acetate; Al, L-alanine; D, dioxane; Gl, L-glutamate; P, L-proline; and S, succinate. The position of the enriched ^{13}C in each detected molecule is indicated by its number (subscript). For succinate, C-2 and C-3 resonances are undistinguishable and are labeled 2,3.

which is deaminated (step 3) or transaminated (step 4) to produce the tricarboxylic acid intermediate 2-ketoglutarate. Because the most abundant excreted end product is L-alanine,

which is the result of an amino group transfer to pyruvate from another amino acid (presumably L-glutamate), L-alanine aminotransferase (EC 2.6.1.2; step 4) is probably the key enzyme for 2-ketoglutarate production. 2-Ketoglutarate is then converted in the tricarboxylic acid cycle to fumarate (steps 5–7), which is reversibly hydrated to malate by the mitochondrial fumarase (step 11) and possibly the cytosolic fumarase (step 12) (13). Theoretically, procyclic trypanosomes can use three different pathways located in different compartments to convert malate into pyruvate, the substrate of L-alanine aminotransferase for L-alanine production: (i) MEm (step 13), (ii) MEc (step 14), and (iii) the cytosolic malate dehydrogenase (EC 1.1.1.37; step 15) associated with glycosomal PEPCK (EC 4.1.1.32; step 16), which is transiently located in the cytosol, and the cytosolic pyruvate kinase (step 17). Alternatively, the latter enzyme may be replaced by the glycosomal pyruvate phosphate dikinase (EC 2.7.9.1; step 18), and steps 15 and 16 may take place in the glycosomes.

Several evidences indicate that the relatively high quantity of excreted L-glutamate is not an artifact of our *in vitro* assay. First, the experiment was conducted with 2×10^7 cells/ml, a cell density that precedes the end of exponential phase. Second, equivalent data were obtained by replacing PBS with the growth medium SDM80 (data not shown). Third, excretion of L-glutamate in both glucose-rich and glucose-depleted media has already been reported (18). Fourth, addition of an excess of unenriched pyruvate (which could theoretically stimulate L-glutamate transamination by L-alanine aminotransferase) does not significantly affect the rate of L-glutamate excretion (Table 1). In this latter experiment, the percentage of excreted ^{13}C -enriched L-alanine was reduced by ~ 2 -fold compared with the same analysis performed in the absence of pyruvate, confirming that L-alanine is produced from pyruvate.

Using L-[U- ^{14}C]proline, van Weelden *et al.* (19) reported that CO_2 accounts for some 80% of the end products of L-proline metabolism. However, it was not possible in those experiments to determine which carbon atoms were released in CO_2 . C-4 of L-proline is only released as CO_2 if converted to acetyl-CoA that enters the tricarboxylic acid cycle (steps 5 and 28). In our experiments using L-[4- ^{13}C]proline, we detected no HCO_3^- (an NMR-detectable surrogate for CO_2 that presents a peak at 163 ppm; data not shown), which indicates that acetyl-CoA derived from L-proline does not significantly enter the tricarboxylic acid cycle.

L-Proline Metabolism in Glucose-rich Medium—To investigate L-proline metabolism in glucose-rich conditions, we determined the ^{13}C -enriched end products of L-[4- ^{13}C]proline degradation excreted from the EATRO1125.T7T cell lines maintained in the presence of 6.15 mM D-glucose. The parasite excretes mainly L-glutamate (50.2%) and succinate (39.2%), with only small amounts of L-alanine (4.4%), acetate (3.8%), β -hydroxybutyrate (1.3%), and lactate (0.7%) also produced (Fig. 1B and Table 1).

According to classical metabolic charts (see Fig. 2), proline-derived succinate is produced by the tricarboxylic acid cycle enzyme succinyl-CoA synthetase (EC 6.2.1.5; step 6). However, we previously reported that the FRDg (step 9) accounts for approximately two-thirds of succinate excreted from D-glucose metabolism (12). To determinate the possible role of FRDg in

TABLE 1

Excreted end products of L-[4-¹³C]proline metabolism by procyclic *T. brucei* cell lines

Cell line ^a	Medium ^b	<i>n</i> ^c	¹³ C-enriched excreted molecules ^d									
			Alanine	Succinate	Glutamate	Acetate	β-Hydroxybutyrate	Malate	Fumarate	Aspartate	Lactate	Total
nmol/h/mg protein												
EATRO1125.T7T	PBS	3	318 ± 17	3 ± 1.3	140 ± 9	17 ± 8	16 ± 2.2	1.3 ± 0.9	ND ^e	3.7 ± 0.9	ND	498 ± 37
EATRO1125.T7T ^f	PBS	2 ^g	260	2	95	17	33	ND	ND	2.0	ND	410
EATRO1125.T7T	PBS + Glu	3	10 ± 2	94 ± 20	120 ± 26	9 ± 3	3 ± 1.2	0.6 ± 0.4	ND	0.6 ± 0.5	1.8 ± 0.6	238 ± 44
EATRO1125.T7T ^h	PBS + Glu	3 ^g	18 ± 3	176 ± 14	145 ± 19	14 ± 4	ND	1.1 ± 0.9	ND	0.7 ± 0.5	4.5 ± 3.2	361 ± 19
EATRO1125.T7T	PBS + Pyr	2 ^g	107	ND	197	4	20	ND	ND	ND	4.0	333
<i>RNAi</i> ⁱ FRDg-C2.ni	PBS + Glu	3	53 ± 2	71 ± 3	117 ± 3	24 ± 2	13 ± 4.7	36.6 ± 1.7	6.8 ± 0.3	1.9 ± 0.5	ND	322 ± 10
<i>RNAi</i> ⁱ FRDg-C2.i	PBS + Glu	3	53 ± 4	52 ± 4	113 ± 9	25 ± 3	16 ± 1.3	40.8 ± 4.0	6.1 ± 0.8	1.0 ± 0.2	0.9 ± 0.6	307 ± 21
<i>RNAi</i> ⁱ FHc-A5.ni	PBS	2	341	28	185	63	11	6.0	ND	ND	ND	630
<i>RNAi</i> ⁱ FHc-A5.i	PBS	2	226	13	133	36	7	7.0	ND	ND	ND	425
<i>RNAi</i> ⁱ FHm-A1.ni	PBS	1	152	12	86	52	ND	ND	ND	3.4	ND	304
<i>RNAi</i> ⁱ FHm-A1.1	PBS	1	93	81	157	26	ND	ND	ND	ND	ND	358
<i>RNAi</i> ⁱ SDH-A1.ni	PBS	2	229	7	85	23	18	4.4	ND	3.9	ND	370
<i>RNAi</i> ⁱ SDH-A1.i	PBS	2	12	296	169	9	ND	ND	ND	ND	ND	486
<i>RNAi</i> ⁱ SDH-A1.i	PBS + Glu	1	2	409	100	13	ND	ND	ND	ND	ND	524
<i>RNAi</i> ⁱ PEPCK-E7.ni	PBS	2	177	10	138	32	11	2.0	ND	6.0	ND	376
<i>RNAi</i> ⁱ PEPCK-E7.i	PBS	3	167 ± 23	12 ± 3	112 ± 32	31 ± 6.5	11 ± 3.9	6.0 ± 2.4	0.8 ± 0.6	10.7 ± 1.8	ND	350 ± 50
<i>RNAi</i> ⁱ PDH-E2.i	PBS	3	421 ± 11	12 ± 1	94 ± 9	ND	ND	3.2 ± 0.4	ND	ND	13 ± 1.6	544 ± 21
¹³ C-enriched excreted molecules												
%												
EATRO1125.T7T	PBS	3	64.2	0.5	28.2	3.2	3.1	0.3	ND	0.5	ND	100
EATRO1125.T7T	PBS	2	64.0	0.4	23.5	3.7	8.2	ND	ND	0.2	ND	100
EATRO1125.T7T	PBS + Glu	3	4.4	39.2	50.2	3.8	1.3	0.3	ND	0.1	0.7	100
EATRO1125.T7T	PBS + Glu	3	5.1	49.0	40.2	4.0	ND	0.3	ND	0.2	1.2	100
EATRO1125.T7T	PBS + Pyr	2	32.2	ND	59.2	1.3	6.0	ND	ND	ND	1.2	100
<i>RNAi</i> ⁱ FRDg-C2.ni	PBS + Glu	3	16.6	22.1	35.8	7.3	4.1	11.4	2.1	0.3	ND	100
<i>RNAi</i> ⁱ FRDg-C2.i	PBS + Glu	3	17.2	16.8	36.7	8.4	5.1	13.2	2.0	0.6	0.3	100
<i>RNAi</i> ⁱ FHc-A5.ni	PBS	2	53.5	4.4	29.1	9.9	2.1	0.9	ND	ND	ND	100
<i>RNAi</i> ⁱ FHc-A5.il	PBS	2	50.9	3.0	31.8	8.5	4.3	1.6	ND	ND	ND	100
<i>RNAi</i> ⁱ FHm-A1.ni	PBS	1	49.7	4.1	28.1	17.1	ND	ND	ND	1.1	ND	100
<i>RNAi</i> ⁱ FHm-A1.1	PBS	1	26.1	22.7	43.8	7.4	ND	ND	ND	ND	ND	100
<i>RNAi</i> ⁱ SDH-A1.ni	PBS	2	61.9	1.8	23.0	6.2	4.7	1.2	ND	1.1	ND	100
<i>RNAi</i> ⁱ SDH-A1.i	PBS	2	2.5	60.8	34.8	1.9	ND	ND	ND	ND	ND	100
<i>RNAi</i> ⁱ SDH-A1.i	PBS + Glu	1	0.3	78.0	19.1	2.6	ND	ND	ND	ND	ND	100
<i>RNAi</i> ⁱ PEPCK-E7.ni	PBS	2	45.5	2.5	35.6	8.3	5.7	0.7	0.1	1.6	ND	100
<i>RNAi</i> ⁱ PEPCK-E7.i	PBS	3	47.3	3.4	30.8	8.6	4.9	1.8	0.2	3.0	ND	100
<i>RNAi</i> ⁱ PDH-E2.i	PBS	3	77.4	2.2	17.3	ND	ND	0.6	ND	0.1	2.4	100

^a .i indicates tetracycline-induced, and .ni indicates non-induced.^b Cells grown in SDM80 medium were incubated in PBS/L-[4-¹³C]proline (PBS), PBS/L-[4-¹³C]proline containing 3.3 mM pyruvate (PBS + Pyr; fifth row), or PBS/L-[4-¹³C]proline containing 3.3 mM glucose (PBS + Glu; fourth row; see Footnote *h*), whereas cells grown in SDM80glu medium were incubated in PBS/L-[4-¹³C]proline containing 3.3 mM D-glucose (PBS + Glu) or PBS/L-[4-¹³C]proline alone (second row; see Footnote *f*).^c *n* indicates the number of experiments for each cell line.^d The results of a single experiment or the means of two to three experiments are presented; the results are shown as the means ± S.D. when three experiments were performed per cell line.^e ND, not detectable.^f The cells were cultivated in the presence of D-glucose (SDM80glu medium), whereas the D-glucose was not present in the incubation buffer (PBS/L-[4-¹³C]proline).^g When only two experiments were analyzed per cell lines, the deviation of the mean (difference between the values divided by 2) was lower than 20% of the mean.^h The cells were cultivated in the absence of D-glucose (SDM80 medium), whereas the D-glucose was present in the incubation buffer (PBS/D-glucose/L-[4-¹³C]proline).

succinate production from L-proline metabolism, the same analysis was conducted on the *RNAi*ⁱFRDg-C2.i RNAi-harboring cell line grown in glucose-rich conditions. Metabolic analyses of this cell line were performed 7 days after tetracycline induction of the RNAi construct, 3 days after FRDg ceased to be detectable by Western blotting (12). In the absence of FRDg, the *RNAi*ⁱFRDg-C2.i cell line continued to excrete large quantities of succinate (Table 1), indicating that most (if not all) proline-derived succinate excreted by procyclic cells is produced in the mitochondrion and not in the glycosomes by FRDg. However, we cannot rule out the possibility that a significant part of succinate is produced by FRDg because the *RNAi*ⁱFRDg-C2.i RNAi-harboring cell line excretes less succinate than the wild-type cells. This is probably the consequence of the increase in L-alanine excretion, which is produced from succinate (Fig. 2 and Table 1). Although, L-proline metabolism is significantly affected in the absence of FRDg, we conclude that most (if not all) of succinate excreted from L-proline metabolism is pro-

duced by the tricarboxylic acid cycle enzyme succinyl-CoA synthetase.

Rapidity of the Metabolic Adaptation—All of the experiments presented above were conducted on the parental or RNAi-harboring procyclic cell lines pre-adapted at least 2 weeks in the experimental conditions (glucose-rich or glucose-depleted conditions). To study the rapidity of the metabolic adaptation, metabolic analyses were conducted on the EATRO1125.T7T procyclic trypanosomes adapted to SDM80 or SDM80glu medium and subsequently incubated in PBS containing L-[4-¹³C]proline with or without D-glucose, respectively. The cells incubated in the absence of D-glucose showed the same pattern of ¹³C-enriched excreted end products, regardless of whether D-glucose was present in the growth medium or not (Table 1, first and second rows). Conversely, the cells incubated with D-glucose showed the same pattern of ¹³C-enriched excreted end products in both growth conditions (Table 1, third and fourth rows). This indicates that the parasite

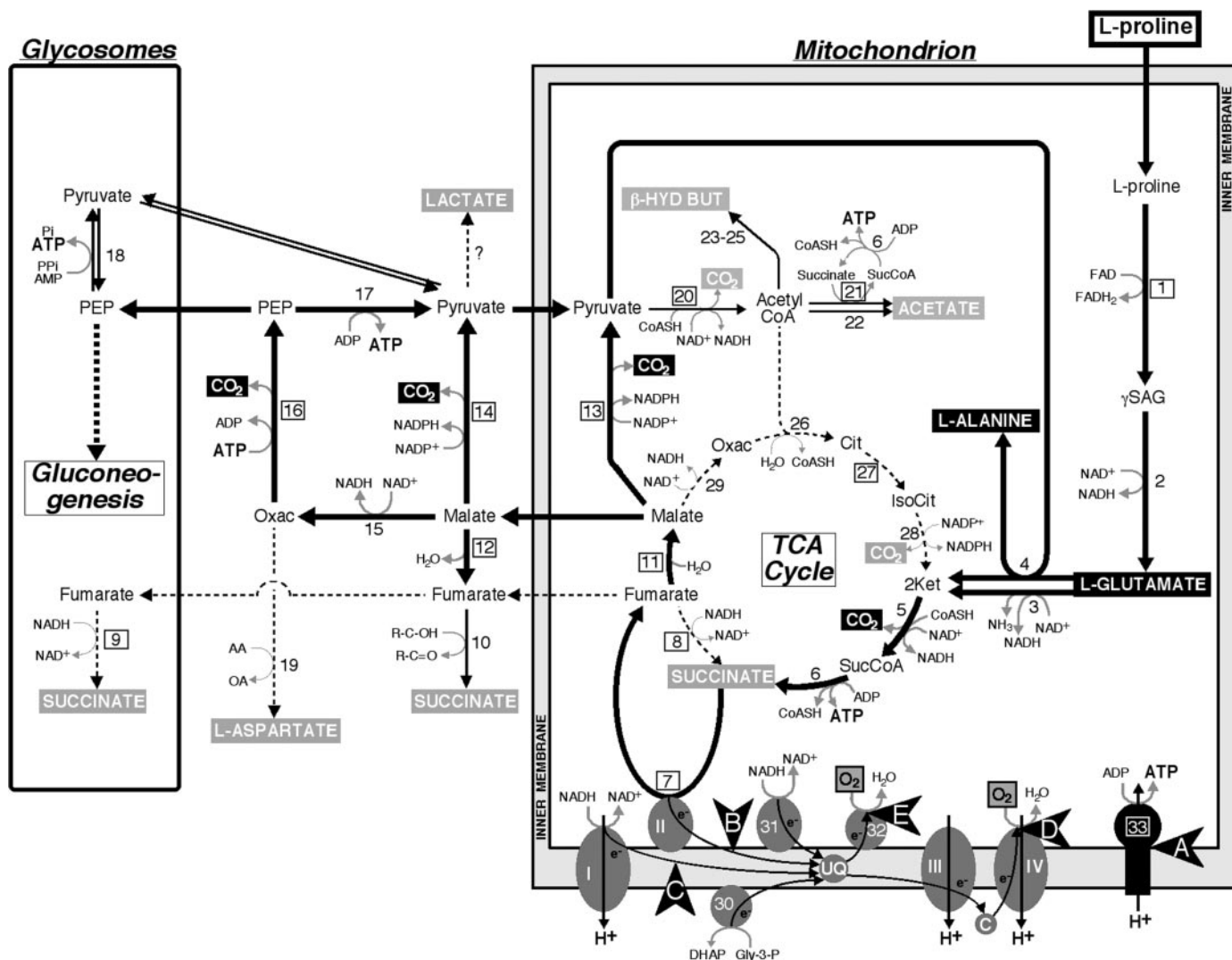


FIGURE 2. **Schematic representation of L-proline metabolism in the procyclic form of *T. brucei* grown in glucose-depleted medium.** Black arrows represent enzymatic steps of L-proline metabolism, and excreted end products are shown as white characters in black boxes (major end products: L-alanine, L-glutamate, and CO₂) or in gray boxes (minor end products: acetate, succinate, lactate, β -hydroxybutyrate (β -HYDBUT), and L-aspartate). For reversible steps, only the presumed or demonstrated direction of the reaction is represented. Dashed arrows indicate steps that are supposed to occur at a background level or not at all under glucose-depleted growth conditions. The enzymes targeted by RNAi or knock-outs are indicated by a boxed number. The enzymatic reaction leading to the production of lactate (possibly from pyruvate) is not known and is indicated by a question mark. The arrowheads indicate the electron transfer steps of the respiratory chain inhibited by malonate (arrowhead B), rotenone (arrowhead C), potassium cyanide (arrowhead D), and salicylhydroxamic acid (arrowhead E); oligomycin (arrowhead A) is a specific inhibitor of the mitochondrial F₀/F₁-ATP synthase. The glycosomal compartments, the mitochondrial compartments, the tricarboxylic acid cycle (TCA), and gluconeogenesis are indicated. AA, amino acid; C, cytochrome c; Cit, citrate; DHAP, dihydroxyacetone phosphate; Gly-3-P, glycerol 3-phosphate; IsoCit, isocitrate; 2Ket, 2-ketoglutarate; OA, 2-oxoacid; Oxac, oxalacetate; PEP, phosphoenolpyruvate; γ SAG, glutamate γ -semialdehyde; SucCoA, succinyl-CoA; UQ, ubiquinone pool. Enzymes are as follow: step 1, proline dehydrogenase (PRODH); step 2, pyrroline-5-carboxylate dehydrogenase; step 3, glutamate dehydrogenase; step 4, L-alanine aminotransferase (ALAT); step 5, 2-ketoglutarate dehydrogenase complex; step 6, succinyl-CoA synthetase; step 7, succinate dehydrogenase (SDH); complex II of the respiratory chain; step 8, mitochondrial NADH-dependent fumarate reductase (FRDm1); step 9, glycosomal NADH-dependent fumarate reductase (FRDg); step 10, oxidoreductase enzymes such as dihydroorotate dehydrogenase; step 11, mitochondrial fumarase (FHm); step 12, cytosolic fumarase (FHC); step 13, mitochondrial malic enzyme (MEM); step 14, cytosolic malic enzyme (MEC); step 15, cytosolic malate dehydrogenase; step 16, phosphoenolpyruvate carboxykinase (PEPCK); step 17, pyruvate kinase (PYK); step 18, pyruvate-phosphate dikinase (PPDK); step 19, L-aspartate aminotransferase; step 20, pyruvate dehydrogenase complex (PDH); step 21, acetate:succinate CoA-transferase (ASCT); step 22, unknown enzyme; steps 23–25, possibly hydroxymethylglutaryl-CoA synthase (Tb927.8.6110), hydroxymethylglutaryl-CoA lyase (Tb927.4.2700), and β -hydroxybutyrate dehydrogenase (Tb10.389.1850), respectively; step 26, citrate synthase; step 27, aconitase; step 28, NADP-dependent isocitrate dehydrogenase; step 29, mitochondrial malate dehydrogenase; step 30, FAD-dependent glycerol-3-phosphate dehydrogenase; step 31, rotenone-insensitive NADH dehydrogenase; step 32, alternative oxidase; step 33, F₁/F₀-ATP synthase (complexes I–IV complexes of the respiratory chain).

responds rapidly to the absence or addition of D-glucose in the medium.

SDH Activity Is Essential in the Absence of D-Glucose—In the presence of D-glucose, 89.4% of the excreted end products of metabolism of L-[4-¹³C]proline were succinate and L-glutamate, whereas in the absence of D-glucose, L-alanine was the most abundant excreted molecule (Table 1). It appears there-

fore that conversion of succinate (produced from L-proline metabolism) into L-alanine is required only in the absence of D-glucose. To test this hypothesis, the expression of the enzyme catalyzing the conversion of succinate to fumarate (SDH; Fig. 2, *step 7*) was inhibited by RNAi. The ^{RNAi}SDH-A1 cell line was produced in the EATRO1125.T7T procyclic cell line designed for conditional expression of double-stranded RNA (23).

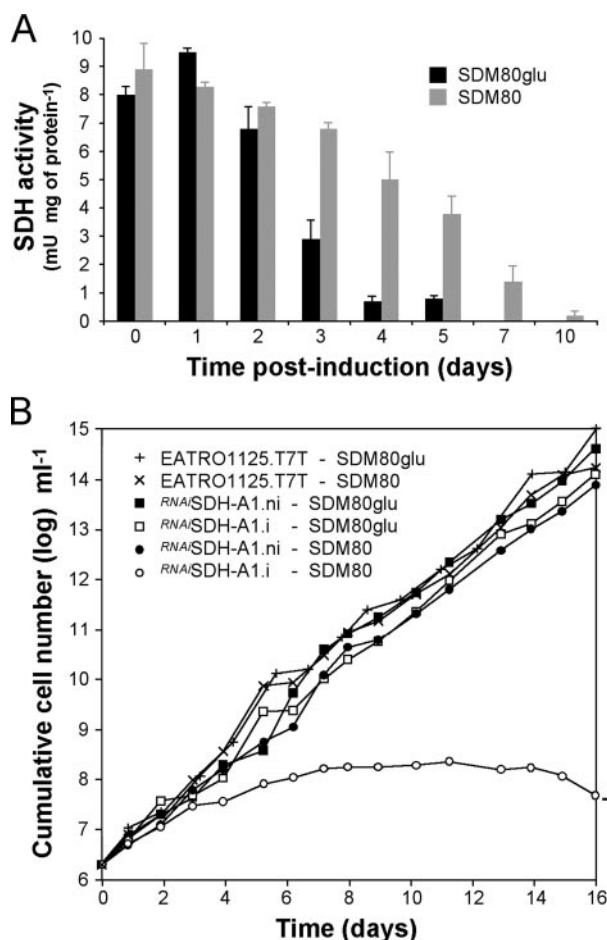


FIGURE 3. Analysis of an SDH-deficient cell line. A shows the SDH activity (milliunits/mg of protein) of the *RNAi*SDH-A1.i *RNAi*-harboring cell line grown in SDM80glu or SDM80 medium in the presence of tetracycline. The SDH activities are normalized with the pyruvate kinase activity measured in the same samples. B shows the growth curves of the EATRO1125.T7T and *RNAi*SDH-A1 procyclic cell lines incubated in SDM80 medium either containing (SDM80glu) or lacking (SDM80) 6 mM D-glucose. Cells were maintained in the exponential growth phase (between 10^6 and 10^7 cells/ml), and cumulative cell numbers reflect normalization for dilution during cultivation. The *RNAi*SDH-A1 cell line was incubated in the presence (.i) or absence (.ni) of tetracycline. The cross indicates that further incubation led to the death of the whole population.

The SDH activity decreased gradually to below detectable levels after 10 days of tetracycline induction, regardless of the amounts of D-glucose in the growth medium (Fig. 3A). It is noteworthy that the enzymatic activity decay was faster in the glucose-rich conditions. The absence of SDH activity in the *RNAi*SDH-A1.i cell line was confirmed by NMR analysis of ¹³C-enriched end products excreted from metabolism of L-[4-¹³C]proline. As expected, the *RNAi*SDH-A1.i *RNAi*-harboring cell line produced mainly L-glutamate and succinate, with only traces of L-alanine, in either the presence or absence of D-glucose (Fig. 1C and Table 1). In contrast, the non-induced *RNAi*SDH-A1.ni cells, as well as the parental cells, excreted mainly L-alanine in the absence of D-glucose (Table 1).

Interestingly, the doubling time of the *RNAi*SDH-A1.i cell line grown in glucose-rich conditions was not affected (Fig. 3B), indicating that a truncated L-proline degradation pathway is adequate for the needs of procyclic trypanosomes when D-glucose is the main carbon source. However, in glucose-depleted

conditions, inhibition of SDH gene expression became lethal (Fig. 3B and Table 2). Conversion of succinate (from L-proline metabolism) to L-alanine is essential for cell viability only in glucose-depleted conditions.

Conversion of Fumarate to Pyruvate in Glucose-depleted Conditions—We previously reported that production of fumarate in the cytosol is essential for the viability of procyclic trypanosomes (13). This intermediary metabolite is the principal electron acceptor for oxidoreductase enzymes, including cytosolic dihydroorotate dehydrogenase, a key enzyme of pyrimidine biosynthesis (41). In glucose-depleted conditions, fumarate is first produced in the mitochondrion by SDH. Part of it can be shuttled between the mitochondrial and cytosolic compartments. Alternatively, if fumarate is poorly exported to the cytosol, part of malate produced from fumarate in the mitochondrion (*step 11*) should reach the cytosol, where it could serve as a substrate for cytosolic fumarase or MEc to produce fumarate or pyruvate, respectively (*steps 12* and *14*). To discriminate between these hypotheses, *RNAi*FH *RNAi*-harboring cell lines were analyzed in glucose-depleted conditions (Tables 1 and 2). As expected, repression of both fumarase genes was lethal (*RNAi*FHc/m-F10.i cell line). In contrast, both the *RNAi*FHc-A5.i and *RNAi*FHm-A1.i cell lines were viable, although their doubling times were significantly affected (increased by 3- and 2.4-fold, respectively), indicating that both cytosolic fumarase (*step 12*) and mitochondrial fumarase (*step 11*) play an important role in L-proline metabolism. The *RNAi*FHm-A1.i cell line showed a 3.4-fold decrease in proline-derived L-alanine excretion, whereas proline-derived succinate efflux increased by 27-fold (Table 1). This clearly indicates that mitochondrial fumarase contributes to the pathway of L-alanine production. In contrast, the rate of L-alanine and succinate production was not significantly affected in the *RNAi*FHc-A5.i cell line, whereas the rate of malate production was increased by 5-fold (this metabolite was not detected in the *RNAi*FHm-A1.i cell line) (Table 1). This suggests that, under these growth conditions, cytosolic fumarase produces fumarate from malate, whereas mitochondrial fumarase functions in the opposite direction. Thus, we propose that malate produced by mitochondrial fumarase in the mitochondrion is more efficiently exported to the cytosol than fumarate, the latter being produced primarily from cytosolic malate by cytosolic fumarase (Fig. 2).

As discussed above, three different routes can theoretically be used to convert malate to pyruvate, which is then converted to the excreted end product L-alanine (Fig. 2). These are MEM (*step 13*), MEc (*step 14*), and/or malate dehydrogenase with PEPCK and pyruvate kinase (*steps 15–17*). To determine which routes are used, we produced and analyzed several tetracycline-inducible *RNAi* cell lines (Table 2). Repression of both malic enzymes using the *RNAi*MEc/m-C3.i cell line in the presence of tetracycline was lethal in SDM80 medium (Table 1), demonstrating that at least one ME is essential for cell viability. Next, we targeted the PEPCK gene (*step 16*). The doubling time of the *RNAi*PEPCK-E7.i cell line in SDM80 medium was increased by 1.5-fold compared with parental cells. NMR analysis of ¹³C-enriched end products excreted from L-[¹³C]proline metabolism showed that the percentage of excreted malate and L-as-

TABLE 2

Effect of enzyme depletions on the doubling time of procyclic *T. brucei* cell lines

Step(s)	Cell line	Clone ^a	Vector	Activity ^b	Protein ^c	Doubling time	
						SDM80glu	SDM80
	EATRO1125.T7T			%		<i>h</i>	
1	<i>RNAi</i> PRODHD ^d	F2.ni	p2T7 ^{Ti}	nd ^e	++	13.5	13.4
1	<i>RNAi</i> PRODHD ^d	F2.i	p2T7 ^{Ti}	nd	ND	WT ^f	WT
7	<i>RNAi</i> SDH	A1.ni	pLew100	80	nd	WT	WT
7	<i>RNAi</i> SDH	A1.i	pLew100	ND	nd	WT	+
8	<i>RNAi</i> FRDm1	G7.ni	pLew79	91	+++	WT	WT
8	<i>RNAi</i> FRDm1	G7.i	pLew79	70	ND	WT	WT
9	<i>RNAi</i> FRDg	C2.ni	pLew79	65	++	WT	WT
9	<i>RNAi</i> FRDg	C2.i	pLew79	35	ND	WT	WT
9/8	<i>RNAi</i> FRDg/m1	B5.ni	pLew79	80	++	WT	WT
9/8	<i>RNAi</i> FRDg/m1	B5.i	pLew79	ND	ND	WT	WT
11	<i>RNAi</i> FHm	A1.ni	pLew100	63	++	18.6	24.7
11	<i>RNAi</i> FHm	A1.i	pLew100	40	ND	19.4	32.4
12	<i>RNAi</i> FHC	A5.ni	pLew100	54	++	18.5	17.3
12	<i>RNAi</i> FHC	A5.i	pLew100	32	ND	32.0	40.1
12/11	<i>RNAi</i> FHC/m	F10.ni	p2T7 ^{Ti} -177	53	++	19.7	nd
12/11	<i>RNAi</i> FHC/m	F10.i	p2T7 ^{Ti} -177	ND	ND	+	+
14/13	<i>RNAi</i> MEc/m	C3.ni	pLew100	54	nd	WT	WT
14/13	<i>RNAi</i> MEc/m	C3.i	pLew100	ND	nd	+	+
16	<i>RNAi</i> PEPCK	E7.ni	pLew100	11	+	17.0	18.5
16	<i>RNAi</i> PEPCK	E7.i	pLew100	ND	ND	18.1	19.9
20	<i>RNAi</i> PDH.E2	.ni	pLew100	100	+++	WT	WT
20	<i>RNAi</i> PDH.E2	.i	pLew100	ND	ND	WT	WT
21	<i>RNAi</i> ASCT	hp4.ni	pLew100	nd	+++	WT	WT
21	<i>RNAi</i> ASCT	hp4.i	pLew100	nd	ND	18.0	22.8
27	Δ aco			nd	nd	17.0	23.0
33	<i>RNAi</i> ATPe-F1 β	.ni	p2T7 ^{Ti} -177	nd	++	17.6	40.1
33	<i>RNAi</i> ATPe-F1 β	.i	p2T7 ^{Ti} -177	nd	ND	23.2	+

^a.i indicates tetracycline-induced, and .ni indicates non-induced.^b Relative enzymatic activities of the targeted protein(s) in the RNAi-harboring cell lines are expressed as a percentage of the activity detected in the parental EATRO1125.T7T cell line.^c Relative amounts of the targeted protein(s) in the RNAi-harboring cell lines are expressed as a relative amount of the protein(s) detected by Western blotting in the parental EATRO1125.T7T cell line.^d Data are from Ref. 18.^e nd, not determined; ND, not detectable.^f The doubling time was similar to that of the parental EATRO1125.T7T cell line.^g +, not viable.

partate was increased by 6-fold compared with parental cells. The accumulation of malate and L-aspartate related to an accumulation of oxalacetate, the substrate of PEPCK (see Fig. 2). In conclusion, at least the PEPCK and one of the ME routes are used by procyclic trypanosomes grown in glucose-depleted medium.

Acetyl-CoA Metabolism—Acetate is a major end product of D-glucose metabolism in procyclic trypanosomes (20–40% of the excreted end products, depending on the analyses) (10, 11). In contrast, L-proline-derived acetate is a minor end product because it accounts for 3–4% of the excreted end products of L-proline metabolism in the glucose-rich and glucose-depleted conditions (Table 1).

It has been previously shown that acetyl-CoA produced in the mitochondrion from D-glucose metabolism does not enter the tricarboxylic acid cycle, even though the enzymes of the cycle are present (11, 19), but is rather converted into the excreted acetate. Similarly, our data provide two lines of evidence suggesting that acetyl-CoA produced from L-proline metabolism does not pass through the tricarboxylic acid cycle. First, as described above, production of ¹³C-enriched HCO₃[−] from L-[4-¹³C]proline was not detected in wild-type cells even in glucose-depleted conditions. Moreover, the doubling time of the Δ aco cell line (Δ aco::NEO/ Δ aco::HYG; kindly provided by M. Boshart) (11), which lacks both aconitase alleles (EC 4.2.1.3; step 27), was not significantly different when grown in glucose-

depleted or glucose-rich conditions (23 versus 17 h, respectively) (Table 2). This indicates that inactivation of the tricarboxylic acid cycle has the same moderate effect on the parasite growth regardless of the D-glucose concentration. Second, low amounts of ¹³C-enriched β -hydroxybutyrate were also excreted from L-[4-¹³C]proline metabolism in both growth conditions (1.3 and 3.1%, respectively), whereas it was not detectable from D-[1-¹³C]glucose metabolism. β -Hydroxybutyrate was previously considered a by-product of acetyl-CoA metabolism (15). Consequently, it appears that the L-proline-derived acetyl-CoA is preferentially converted into β -hydroxybutyrate (in addition to acetate) instead of CO₂ through the tricarboxylic acid cycle.

The relevance of acetyl-CoA production from L-proline was further investigated with the analysis of RNAi-harboring cell lines targeted for PDH (step 20) activities. The *RNAi*PDH-E2.i cell line showed no detectable PDH activity upon tetracycline induction (Table 2), and the protein was not detectable by Western blotting. The ability of the PDH-E2 knockdown cell line to produce acetate was estimated by NMR analysis of ¹³C-enriched end products excreted from L-[4-¹³C]proline metabolism. Acetate was not detectable in the *RNAi*PDH-E2.i cell line, but was produced by all of the other cell lines analyzed so far (Table 1). In addition, β -hydroxybutyrate was not detectable upon tetracycline induction of the *RNAi*PDH-E2.i cell line. To confirm these data, the same experiment was performed using

TABLE 3

Excreted end products of D-[1-¹³C]glucose metabolism by procyclic *T. brucei* cell lines

Cell line ^a	Activity ^b	n ^c	¹³ C-enriched excreted molecules ^d							
			Succinate	Acetate	Lactate	Malate	Fumarate	L-Alanine	Pyruvate	Total
nmol/h/mg protein										
EATRO1125.T7T	31.0 ± 4.0	3	470 ± 88	186 ± 21	55 ± 15	20 ± 1.7	4 ± 1.1	8 ± 2.1	ND ^e	743 ± 124
^{RNAi} PDH-E2.ni	29.6 ± 1.1	3	744 ± 92	203 ± 37	103 ± 25	32 ± 4.1	5 ± 0.8	14 ± 8.5	9 ± 5	1099 ± 150
^{RNAi} PDH-Ei.i	ND	3	261 ± 35	11 ± 2.1	36 ± 6	37 ± 5.9	6 ± 1.6	9 ± 5	16 ± 10	150 ± 57

^a.i indicates tetracycline-induced, and .ni indicates non-induced.^b Enzymatic activities of the targeted protein(s) in the RNAi-harboring and parental EATRO1125.T7T cell lines are expressed as milliunits/mg of protein.^c n is the number of experiments for each cell line.^d The means of three experiments are presented; the results are shown as the means ± S.D.^e ND, not detectable.

D-[1-¹³C]glucose as the carbon source because the rate of acetate production was ~10-fold higher when using D-glucose as the carbon source compared with L-proline (Table 3). The rate of acetate production was reduced by 95% in the tetracycline-induced compared with non-induced ^{RNAi}PDH-E2.ni cell line (11 versus 203 nmol/h/mg of protein), and the production of pyruvate, the PDH substrate, was significantly increased. Thus, the production of acetyl-CoA from L-proline is almost completely abolished in the ^{RNAi}PDH-E2.i RNAi-harboring cell line. Interestingly, the absence of PDH activity did not affect the doubling time of the parasite (Table 2), indicating that acetyl-CoA production from D-glucose and L-proline is dispensable for procyclic trypanosomes.

We also targeted by RNAi the acetate:succinate CoA-transferase (EC 2.3.1.8; *step 21*) (15), which consumes acetyl-CoA for acetate production. The doubling time of the ^{RNAi}ASCT-hp4.i cell line, which is devoid of acetate:succinate CoA-transferase in Western blots (data not shown), was similar in the absence or presence of D-glucose (22.8 versus 18 h, respectively).

Oxidative Phosphorylation Is not Essential in Glucose-Rich Conditions—It was previously shown that the mode of ATP production by procyclic trypanosomes (substrate level phosphorylation versus oxidative phosphorylation) depends primarily on D-glucose availability (4, 18, 26, 42). Indeed, as shown in Fig. 4A, the EATRO1125.T7T procyclic cells grown in a glucose-depleted medium were 3000-fold more sensitive to oligomycin, a specific inhibitor of the mitochondrial F₀/F₁-ATP synthase (*step 33*), than the same cells grown in the presence of D-glucose. This conclusion has recently been questioned (43) because the doubling time of cells grown in glucose-rich medium is affected by oligomycin used at high concentrations (26). However, because any chemical can have nonspecific effects when used at relatively high concentrations, we generated an RNAi-harboring cell line to directly address this question. The ^{RNAi}ATPε-F1β cell line loses expression of ATPε-F1β, which is directly involved in ATP synthesis by the mitochondrial F₀/F₁-ATP synthase (44). The ^{RNAi}ATPε-F1β.i cells grown in the glucose-depleted medium died 7 days after tetracycline induction (Fig. 5A), although ATPε-F1β was still detected by Western blotting (Fig. 5B). In contrast, the doubling time of the ^{RNAi}ATPε-F1β.i cell line was only slightly affected in the glucose-rich medium (23.2 versus 13.5 h), whereas ATPε-F1β was not detectable by Western blotting (Fig. 5). Therefore, sensitivity to loss of the mitochondrial F₀/F₁-ATP synthase depends on the level of D-glucose. This finding clearly indicates that oxidative phosphorylation is not

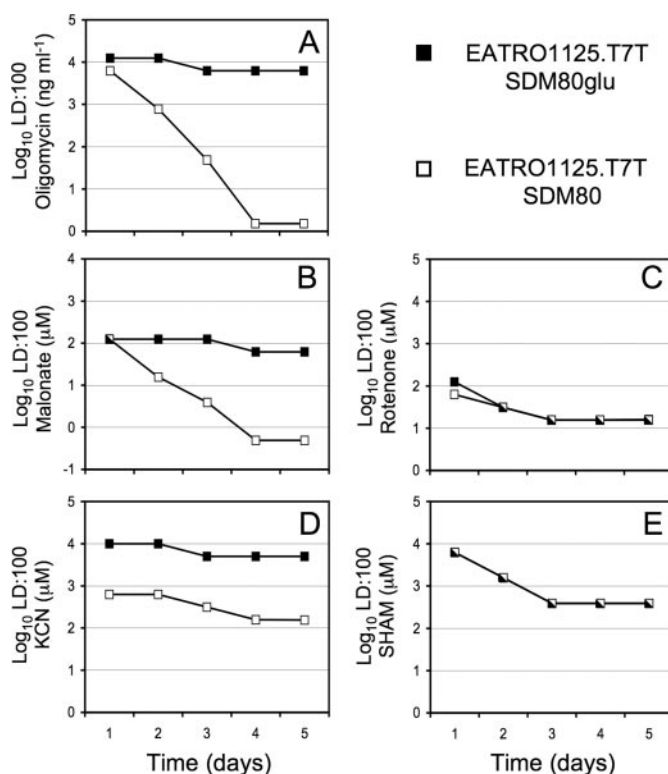


FIGURE 4. **Effect of metabolic drugs on cell viability.** The EATRO1125.T7T cells were inoculated (2×10^6 cells/ml) into SDM80 medium either containing (SDM80glu) or lacking (SDM80) 6 mM glucose in the presence of decreasing concentrations of oligomycin (A), malonate (B), rotenone (C), KCN (D), or salicylhydroxamic acid (SHAM; E). The drug concentration required to kill all of the cells is shown as a function of days of incubation after drug addition (LD₁₀₀). A logarithmic scale is used for the drug concentration. The values represent the means of three to six different experiments with an S.D. ranging below 30% of the values.

essential for the procyclic trypanosomes in the presence of D-glucose, whereas in the absence of this sugar, it is.

Effect of Metabolic Inhibitors on Cell Viability—To test whether the effect of other classical metabolic inhibitors on cell viability is also dependent on the carbon source, the minimum concentration of the drug capable of killing 100% of the EATRO1125.T7T procyclic cell line (LD₁₀₀) was determined for cells grown in the presence or absence of D-glucose. Malonate, a specific inhibitor of electron transfer from SDH (complex II of the respiratory chain; *step 7*) to the ubiquinol pool, was 130-fold more active against procyclic cells grown in the glucose-depleted medium (Fig. 4B), consistent with data obtained with the ^{RNAi}SDH-A1.i RNAi-harboring cell line (Fig. 3). Similarly, the parasite was 32-fold more sensitive to KCN, a specific

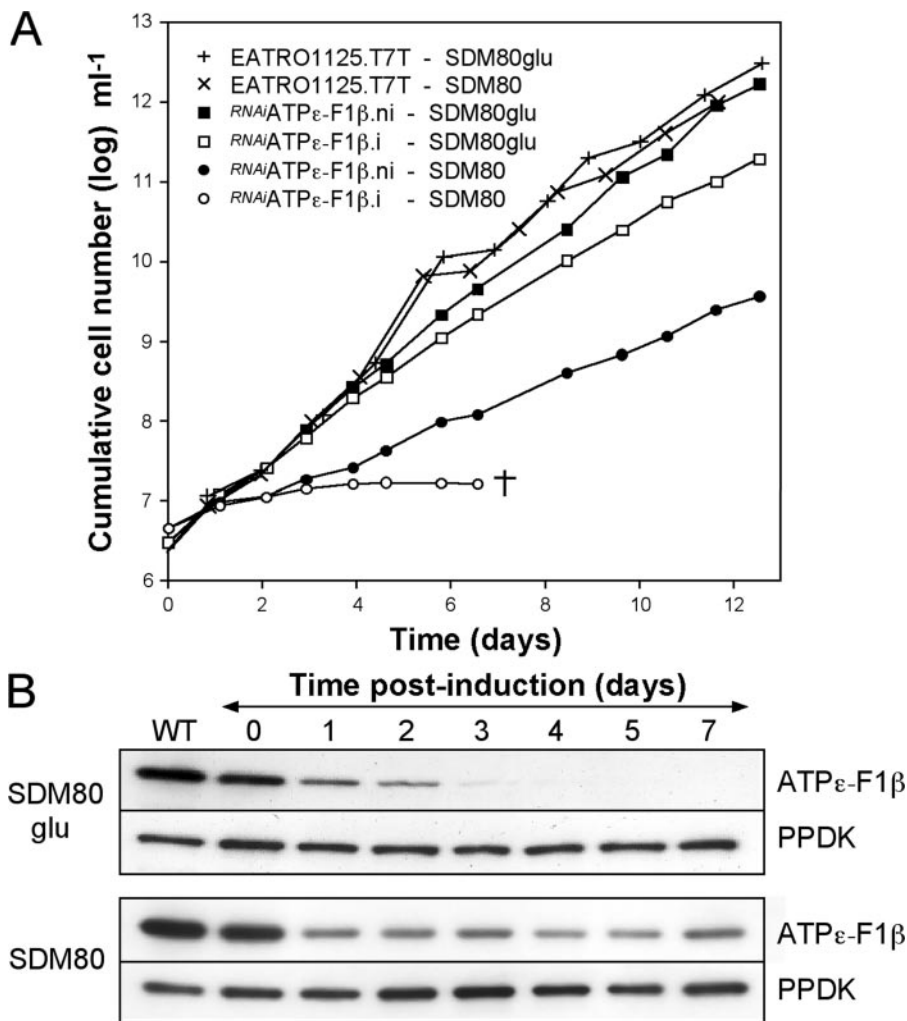


FIGURE 5. Analysis of an F_0/F_1 -ATP synthase-deficient cell line. A shows the growth curves of the EATRO1125.T7T and RNAi ATP ϵ -F1 β RNAi-harboring procyclic cell lines incubated in SDM80 medium either containing (SDM80glu) or lacking (SDM80) 6 mM D-glucose. Cells were maintained in the exponential growth phase (between 10^6 and 10^7 cells/ml), and cumulative cell numbers reflect normalization for dilution during cultivation. The RNAi ATP ϵ -F1 β cell line was incubated in the presence (i) or absence (ni) of tetracycline. The cross indicates that further incubation led to the death of the whole population. B shows Western blot analysis of the ATP ϵ -F1 β and pyruvate phosphate dikinase (PPDK) proteins in the parental (wild-type (WT)) and RNAi ATP ϵ -F1 β .i cell lines grown in SDM80glu or SDM80 medium in the presence of tetracycline.

inhibitor of the terminal oxidase (complex IV of the respiratory chain), when grown in glucose-depleted medium. However, the effects of salicylhydroxamic acid or rotenone, which selectively inhibits the alternative oxidase (step 32) or complex I (rotenone-sensitive NADH dehydrogenase) of the respiratory chain respectively, had the same effect on cell growth regardless of the amount of D-glucose in the medium (Fig. 4, C and E).

DISCUSSION

Procyclic forms of *T. brucei* are parasitic protozoa whose normal habitat is the midgut of the tsetse fly, an environment in which D-glucose and other sugars are usually scarce. However, only recently have culture media that reflect this scarcity of D-glucose been developed to culture these parasites, the traditional media instead being rich in D-glucose (16–19, 45). We have previously shown that the consumption of L-proline, the principal carbon and energy source available in tsetse fly hemolymph, is down-regulated on the order of 6-fold in two different

laboratory-adapted trypanosome cell lines (EATRO1125 and 427) (18). In the absence of D-glucose, however, L-proline has been shown to be the principal source of carbon and energy for the trypanosome (18). In this study, we determined the L-proline metabolism pathways in both growing conditions using a combination of NMR metabolic analyses and 13 RNAi-harboring or knock-out cell lines affecting L-proline catabolism (Fig. 2). We also showed that the L-proline metabolism pathway is considerably reduced in glucose-rich conditions (Fig. 6).

When D-glucose was abundant, succinate and L-glutamate represented the principal excreted end products of L-proline metabolism, which is consistent with previous data (11, 19). However, in glucose-depleted conditions, procyclic *T. brucei* converted succinate further to L-alanine (the main excreted end product, with L-glutamate being also excreted). A previous analysis performed on procyclic *Trypanosoma congolense* (a parasite related to *T. brucei*) grown in glucose-depleted conditions showed that in this species, too, L-glutamate (59%) and L-alanine (34%) are the main end products excreted from L-proline metabolism (46). However, the only previous analysis addressing this question in *T. brucei* (using the TREU927 strain) reported that succinate, CO₂, and

acetate are the only excreted end products from L-[U-¹⁴C]proline metabolism under conditions of both glucose abundance and glucose restriction (19). It seems probable that the TREU927 procyclic strain used in this analysis (19), which also increases consumption of L-proline by only ~2-fold as opposed to the 6-fold we noted in the EATRO1125 strain, has a different capacity to adapt to changes in D-glucose abundance than the strains we have studied here.

L-Proline is the principal energy source for tsetse fly flight muscle (7, 40, 47). It is produced by the insect's fat body from L-alanine and acetyl-CoA, the latter deriving from fatty acid, D-glucose, or amino acid metabolism. In flight muscles, ATP is produced by conversion of L-proline to L-alanine. Consequently, L-proline is the main carbon source present in the hemolymph of the insect vector and is the primary carbon source available for the insect parasites. Interestingly, we showed here that L-proline metabolism in procyclic trypanosomes grown in the absence of D-glucose mimics that seen in

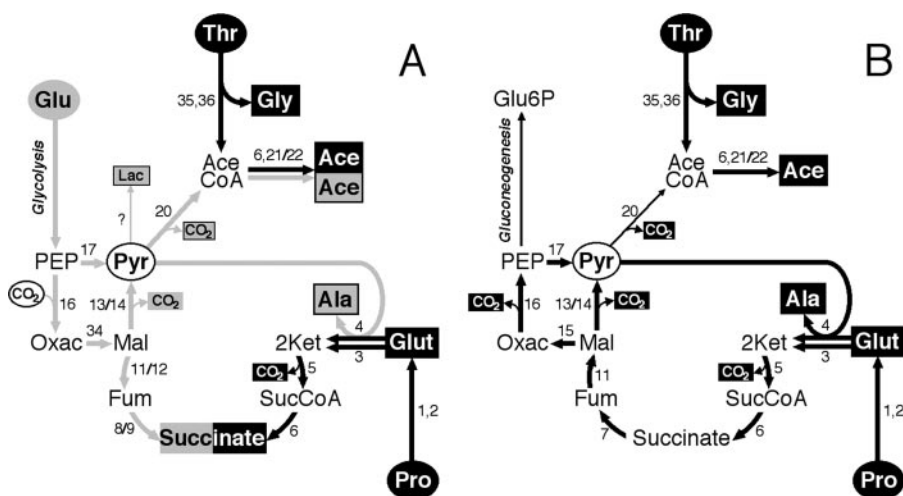


FIGURE 6. Schematic representation of carbon source metabolism in the procyclic form of *T. brucei* grown in glucose-rich (A) and glucose-depleted (B) media. Carbon sources consumed in glucose-rich and glucose-depleted media (18, 26) are indicated in a white (pyruvate), gray (D-glucose), or black (L-proline and L-threonine) oval. Metabolic compartments are not indicated. Enzymatic steps of L-proline/L-threonine and D-glucose/pyruvate metabolisms are represented by black and gray arrows, respectively. Excreted end products are shown in white characters in black boxes (L-proline and L-threonine metabolism) or gray boxes (D-glucose and pyruvate metabolism). Ace, acetate; AceCoA, acetyl-CoA; Ala, L-alanine; Fum, fumarate; Glu, D-glucose; Glu6P, glucose 6-phosphate; Glut, L-glutamate; Gly, L-glycine; 2Ket, 2-ketoglutarate; Lac, lactate; Mal, malate; Oxac, oxalacetate; PEP, phosphoenolpyruvate; Pro, L-proline; Pyr, pyruvate; SucCoA, succinyl-CoA; Thr, L-threonine. For enzyme names, see Fig. 2. Step 34, glycosomal malate dehydrogenase; step 35, L-threonine dehydrogenase; step 36, acetyl-CoA:glycine C-acetyltransferase. The slashes separate enzymes or isoenzymes of different routes.

flight muscle inasmuch that L-alanine is also the main end product.

L-Proline metabolism produces several reducing equivalents (Fig. 2, steps 1–3, 5, and 7), which can be reoxidized by the respiratory chain while contributing to the proton gradient that can be used for ATP production by oxidative phosphorylation. It is still unclear how procyclic trypanosomes reoxidize mitochondrial-reducing equivalents such as NADH. Two NADH dehydrogenases are associated with the mitochondrial inner membrane of this parasite, a rotenone-sensitive NADH:ubiquinone oxidoreductase (complex I of the respiratory chain) (48) and a rotenone-insensitive NADH dehydrogenase, which oxidizes NADH without proton translocation across the membrane (49). The role of complex I in energy metabolism of procyclic trypanosomes is uncertain (50, 51). For instance, complex I activity observed in mitochondrial fractions shows an unusually low sensitivity to rotenone; hence, the relatively high quantity of rotenone required to kill the parasite has been proposed to arise from nonspecific binding of this metabolic poison to other electron carriers (52). According to the *T. brucei* metabolic schemes (18), 10-fold more NADH is produced per mol of L-proline than per mol of D-glucose. The fact that we see no increased sensitivity to rotenone in the parasites indicates that the increased NADH production in mitochondria using L-proline is not matched by an increase in complex I activity. The role of complex I in NADH oxidation warrants further study. Turrens and co-workers (51, 53) proposed that part of the succinate produced from L-proline and D-glucose metabolism may be involved in a cycle involving both the SDH and FRD mitochondrial activities. In this model, fumarate accepts electrons from NADH (FRD activity) to be converted to succinate, which is oxidized back to

fumarate by complex II of the respiratory chain (SDH). Electrons are then transferred via the quinone pool and the respiratory chain to molecular oxygen. Although hypothetical, this model is supported by the presence in the mitochondrion of the procyclic trypanosomes of both an FRD activity encoded by *FRDm1* (12) and an SDH activity (53). Clearly, the maintenance of the mitochondrial redox balance in procyclic trypanosomes is an open question that requires further comprehensive analyses of each working hypothesis. Although of unaltered sensitivity to rotenone, procyclic cells grown in glucose-depleted conditions are significantly more sensitive to malonate (130-fold) and oligomycin (3000-fold) compared with cells grown in high levels of D-glucose, emphasizing the increased importance of the respiratory chain to these cells (Fig. 4).

The bloodstream and procyclic forms of *T. brucei* use different strategies to produce ATP and to generate the proton gradient required to maintain essential mitochondrial functions. In the bloodstream forms, the proton gradient is generated by the mitochondrial F_0/F_1 -ATP synthase (step 33) using ATP produced from the glycolytic pathway (44, 54, 55). In contrast, it is widely accepted that in procyclic trypanosomes, the respiratory chain produces a proton gradient, which is used primarily to produce ATP by oxidative phosphorylation (43). Here, we confirmed that this is true when the cells are grown in glucose-depleted conditions. Indeed, a partial inhibition of the expression of the F_1 complex (β subunit) of the F_0/F_1 -ATP synthase is lethal when amino acids are the only carbon sources available. Because amino acid metabolism produces low amounts of ATP by substrate level phosphorylation, the essential role of the F_0/F_1 -ATP synthase in these growth conditions must be in the synthesis of ATP from the mitochondrial proton gradient, the latter being generated by the respiratory chain from the abundant redox equivalents produced by L-proline metabolism (see Fig. 2). However, the situation is different when procyclic trypanosomes are grown in glucose-rich conditions, where we observed that the F_0/F_1 -ATP synthase activity is not essential. In the presence of D-glucose, ATP is therefore produced primarily by substrate level phosphorylation, as proposed previously (4, 18, 26, 42, 56), with the consequence that respiratory chain and F_0/F_1 -ATP synthase activities are down-regulated under these growth conditions. A second implication from this work relates to the production of the proton gradient by the respiratory chain. The involvement of D-glucose metabolism is negligible because net NADH production in the mitochondrion does not occur using this substrate (the mitochondrial NADH produced by PDH being consumed by mitochondrial FRD, thus maintaining a balance) (12).

However, as mentioned above, L-proline (as well as L-threonine) (4) metabolism produces reducing equivalents that can feed the respiratory chain. We propose that in the presence of D-glucose, amino acid metabolism provides enough reducing equivalents to generate, through the respiratory chain, the proton gradient required to maintain mitochondrial function. Consequently, under these conditions, the F_0/F_1 -ATP synthase is not necessary to produce or consume the proton gradient, as demonstrated experimentally.

Procyclic trypanosomes prefer to consume D-glucose over L-proline, with D-glucose inducing an ~6-fold reduction of the rate of L-proline consumption (18). Here, we have shown that D-glucose also promotes a reduction of the L-proline metabolic pathway and a switch from ATP production by oxidative phosphorylation to substrate level phosphorylation. Two additional lines of evidence illustrate the finesse of these metabolic adaptations. First, the rate of L-proline consumption, as well as the degree of oligomycin sensitivity, depends on the D-glucose concentration in the growth medium (60). Second, RNAi FRDg and RNAi FRDg/m1 RNAi-harboring cell lines maintained in glucose-rich conditions show a reduced rate of D-glucose consumption compensated by an increased rate of L-proline consumption (10, 12). Interestingly, these cell lines show an intermediary sensitivity to oligomycin (LD_{100} = 98 and 47 ng/ml, respectively) compared with the parental cells grown in glucose-rich and glucose-depleted conditions (LD_{100} = 6250 and 2 ng/ml, respectively).³ The metabolic control exerted by D-glucose seems to take place primarily at the metabolic level because this metabolic switch is fast. Indeed, cells grown in glucose-rich medium switch to L-proline metabolism at the most in 1 h (Table 1, second row). Similarly, repression of L-proline metabolism by D-glucose addition is observed after an equivalent delay (Table 1, fourth row). The mechanisms of these metabolic adaptations are not known and are currently under investigation.

In conclusion, procyclic trypanosomes clearly prefer D-glucose over L-proline when both carbon sources are available, although their natural habitat is D-glucose-free. Adaptation of L-proline metabolism to D-glucose metabolism (and vice versa) is a fast, fine, and stepwise process, highlighting the subtleties of the metabolic control elaborated by this parasite. We also observed that long-term culture in glucose-depleted conditions did not affect the activity of all of the glycolytic enzymes.³ This illustrates that procyclic trypanosomes maintain their high glycolytic potentiality even in the absence of D-glucose. It would be interesting to determine whether this capability is also maintained in other *T. brucei* insect stages, including the epimastigotes, which are present in the salivary glands of the insects. Unfortunately, *T. brucei* epimastigotes have not been adapted to *in vitro* culture and thus are not available for biochemical analyses. This question could be addressed in *T. congolense* because its epimastigote forms can be grown *in vitro* after differentiation from procyclic cells (57, 58). The recent development of the RNAi tools in this African trypanosome (59) and its ongoing genome project (www.genedb.org/genedb/tcongolense/) will strengthen the development of this interesting trypanosome model.

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