Fumarate Is an Essential Intermediary Metabolite Produced by the Procyclic Trypanosoma brucei*

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Virginie Coustou†, Marc Biran§, Sébastien Besteiro†, Loïc Rivière‡, Théo Baltz‡, Jean-Michel Franconi§, and Frédéric Bringaud‡

From the †Laboratoire de Génomique Fonctionnelle des Trypanosomatides, UMR-5162 CNRS and ‡Résonance Magnétique des Systèmes Biologiques, UMR-5536 CNRS, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux, France

The procyclic stage of Trypanosoma brucei, a parasitic protist responsible for sleeping sickness in humans, converts most of the consumed glucose into excreted succinate, by succinic fermentation. Succinate is produced by the glycosomal and mitochondrial NADH-dependent fumarate reductases, which are not essential for parasite viability. To further explore the role of the succinic fermentation pathways, we studied the trypanosome fumarases, the enzymes providing fumarate to fumarate reductases. The T. brucei genome contains two class I fumarase genes encoding cytosolic (FHc) and mitochondrial (FHm) enzymes, which account for total cellular fumarase activity as shown by RNA interference. The growth arrest of a double RNA interference mutant cell line showing no fumarase activity indicates that fumarases are essential for the parasite. Interestingly, addition of fumarate to the medium rescues the growth phenotype, indicating that fumarate is an essential intermediary metabolite of the insect stage trypanosomes. We propose that trypanosomes use fumarate as an essential electron acceptor, as exemplified by the fumarate dependence previously reported for an enzyme of the essential de novo pyrimidine synthesis (Takashima, E., Inaoka, D. K., Osanai, A., Nara, T., Odaka, M., Aoki, T., Inaka, K., Harada, S., and Kita, K. (2002) Mol. Biochem. Parasitol. 122, 189–200).

Fumarases, also called fumarate hydratases (FHs), are ubiquitous enzymes, which catalyze the stereospecific reversible hydration of fumarate to malate. Most prokaryotes and eukaryotes express two isoforms of fumarases. In eukaryotes, the mitochondrial isoform performs this reaction as part of the tricarboxylic acid cycle and as such is central to aerobic respiration. The cytosolic isofom, as exemplified by Saccharomyces cerevisiae, is thought to be involved in the metabolism of fumarate, which is produced in the cytosol by a number of reactions (1). Two distinct classes of fumarases, class I and class II, have been so far identified, with no obvious amino acid sequence homology, although they both belong to a superfamily of metabolic enzymes that are functionally related (2–4). Class II fumarases (or fumC enzymes) are homotetrameric, thermostable, iron-independent enzymes with a molecular mass of 200 kDa. They occur in several bacteria (5) and in eukaryotes, such as fungi (6), mammals (7), and higher plants (8). In contrast, class I fumarases (including similar fumA and fumB enzymes) are homodimeric, thermostable, iron-sulfur-containing enzymes of ~120 kDa. Class I fumarases occur primarily in bacteria, including Escherichia coli (9) and are also present in a few unicellular eukaryotes. The so far available data about eukaryotic class I fumarases are restricted to sequencing projects. Here, we analyzed the metabolic role(s) of both class I fumarase isoforms expressed in Trypanosoma brucei, a protozoan parasite belonging to the trypanosomatid family.

Several trypanosomatid species cause serious diseases in humans such as sleeping sickness (T. brucei), Chagas disease (Trypanosoma cruzi), and leishmaniasis (Leishmania spp.) (10, 11). Recently, the genome sequencing projects of T. brucei (927 strain) (12), T. cruzi (CL brener strain) (13), and Leishmania major (Friedlin strain) (14) have been completed. Among these species, T. brucei has become the favorite model to study metabolism and other processes shared with other trypanosomatid species. Indeed, RNA interference (RNAi) has been developed in a very powerful reverse genetic tool for T. brucei, by allowing this parasite to inhibit specifically the expression of a target gene (15), but it failed to be functional in T. cruzi and Leishmania spp. (16, 17). T. brucei differentiates into several adaptive forms during its life cycle: the bloodstream forms in the mammalian host and the procyclic form in the midgut of the tsetse fly vector. Both can easily be kept in axenic cell culture and genetically manipulated.

All trypanosomatids analyzed so far are characterized by aerobic fermentation of glucose, with excretion of partially oxidized end products (18–22). The energy metabolism of the T. brucei bloodstream forms is unique among trypanosomatids. Indeed, they rely entirely on glycolysis (which takes place in glycosomes, peroxisome-like organelles) with pyruvate as the main excreted end product, although the mitochondrion plays a minor role (18, 23). In contrast, the other adaptive forms of trypanosomatids, exemplified by the T. brucei procyclic form, have a more elaborate...
energy metabolism, with several excreted end products, such as succinate, acetate, and lactate (21, 22). The \textit{T. brucei} procyclic form contains a functional mitochondrial electron-transport chain that allows the generation of a proton gradient used for several cellular processes, including ATP generation by the mitochondrial \(F_{o}/F_{1}\)-ATP synthase (oxidative phosphorylation). However, oxidative phosphorylation is not essential when glucose is the major carbon source and most of the required ATP synthesis is provided by substrate level phosphorylation (24–26). Interestingly, the tricarboxylic acid cycle is not used as a \textit{bona fide} cycle, i.e. to convert acetyl-CoA into carbon dioxide, although all the enzymes of the cycle are expressed in the procyclic form grown in glucose-rich medium (27). Instead, acetyl-CoA is converted into acetate (28, 29), which represents 20–40% of the excreted end products from the glucose metabolism by the procyclic trypomastigotes. According to the current model, succinate is the main excreted end product (60–70%) (27, 30); it is produced in both the glycosomes and the mitochondrion, by the so-called succinic fermentation pathway (21, 22, 30–32). About half of the phosphoenolpyruvate (PEP) produced in the cytosol re-enters the glycosomes to be converted into malate by PEP carboxykinase and malate dehydrogenase. Malate is then converted into succinate by fumarases and NADH-dependent fumarate reductases (FRD). Approximately two-thirds of the succinate is produced in the glycosomes by a glycosomal FRD isoform (FRDg) (30), whereas a mitochondrial FRD isoform (FRDm1) produces the remaining succinate (32). This model would also predict that malate is exchanged between the glycosomal and mitochondrial compartments, and fumarase isoforms are present in both compartments to provide both FRD isoforms with fumarate (32). To further study these unusual succinic fermentation pathways, we determined the subcellular location of the fumarases expressed in the procyclic trypomastigotes and analyzed glucose metabolism and growth phenotypes of fumarase mutants.

**EXPERIMENTAL PROCEDURES**

\textbf{Trypanosome and Glycosome Preparation—}The procyclic form of \textit{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\(^{-1}\) hemin (33). The bloodstream form of \textit{T. brucei} AnTat1 was grown in rats and isolated by DEAE ion exchange chromatography, as previously described (34). Glycosomes were purified by isopycnic centrifugation from the procyclic (EATRO1125) form of \textit{T. brucei} as described (35), after homogenizing the cells in STE buffer (250 mM sucrose, 25 mM Tris, pH 7.4, 1 mM EDTA) with silicon carbide as grinding material (36).

\textbf{Phylogenetic Analysis—}The amino acid sequences of class I and class II fumarases were aligned using the multiple alignment option in CLUSTAL X (37), followed by minor manual adjustments using MacClade version 4.06 (Sinauer Associates, Inc.). Phylogenetic trees were generated by the neighbor-joining and maximum parsimony heuristics methods as implemented in PAUP version 4.0b10 (Sinauer Associates, Inc.), using default parameters. Bootstrapping was also carried out using PAUP. The accession numbers of the various sequences used in this study are as follows: 1) ZP_00417946; 2) YP_261882; 3) AAM36330; 4) NP_890615; 5) CAH37820; 6) AAF41965; 7) NP_717819; 8) ZP_00752568; 9) NP_066688; 10) NP_106657; 11) BAB04613; 12) BAD74711; 13) NP_811169; 14) AAQ66471; 15) NP_756975; 16) YP_153174; 17) AAP17105; 18) ZP_00794354; 19) YP_385514; 20) NP_772436; 21) NP_949213; 22) NP_699791; 23) ZP_00267671; 24) BAC70929; 25) AAW26769; 26) XP_650763; 27) XP_817100; 28) XP_804521; 29) XP-828580; 30) CAJ04118; 31) XP_814517; 32) XP_821204; 33) XP_825462; 34) XP_847862; 35) CAI75315; 36) EAN30814; 37) XP_678794; 38) XP_725604; 39) XP_745408; 40) NP_704811; 41) ZP_00728590; 42) YP_150640; 43) ZP_00719847; 44) P93033; 45) CAA97997; 46) AAL90297; and 47) NP_000134.2.

\textbf{Inhibition of FH Gene Expression by RNAi—}The inhibition by RNAi of \(F_{H}\) and/or \(F_{Hm}\) expression in the procyclic forms (15) was performed by expression of stem-loop “sense/antisense” molecules of the targeted sequences (38) introduced in the pLew100 expression vector (kindly provided by E. Wirtz and G. Cross) (39). Construction of the pLew-FHc-SAS and pLew-FHm-SAS plasmids, which target the \(FHm\) gene (from position 1121 to 1668 bp) and the \(Fhc\) gene (from position 1114 to 1605 bp), respectively, was performed as described (25, 28, 30, 32, 38). Briefly, PCR-amplified 531- or 588-bp fragments containing the antisense \(Fhc\) or \(Fhm\) sequences (458 or 492 bp of target sequence, plus 40 or 72 bp used as a spacer to form the loop between the annealing sense and antisense sequences, respectively) and the restriction sites added to the primers were inserted in the HindIII and BamHI restriction sites of the pLew100 plasmid. Then a PCR-amplified 525- or 482-bp fragment containing the sense \(Fhc\) or \(Fhm\) sequences was inserted, upstream of the antisense sequence, using HindIII and Xhol restriction sites (Xhol was introduced at the 3’ extremity of the antisense PCR fragment). To target the \(Fhm\) and \(Fhc\) genes simultaneously, we produced by overlapping PCR chimerical sense and antisense molecules composed of the DNA fragments used to target \(Fhc\) and \(Fhm\). For the chimeric sense molecule, we generated by PCR an \(Fhc\)-S fragment flanked by HindIII (5’-end) and the first 20 bp of the \(Fhc\)-S fragment (3’-end), and an \(Fhc\)-S fragment flanked by the first 20 bp of the \(Fhc\)-S fragment (5’-end) and \(Xhol\) (3’-end). Then, the sense chimeric fragment was produced by overlapping PCR, with the same 5’ and 3’ primers as used to PCR amplify the \(Fhm\)-S and \(Fhc\)-S fragments, respectively, and as templates the gel-purified \(Fhc\)-S and \(Fhc\)-S fragments. The resulting 974-bp PCR fragment is composed of the \(Fhc\)-S fragment (458 bp) followed by the \(Fhc\) fragment (492 bp) and flanked by the HindIII (5’-end) and \(Xhol\) (3’-end) restriction sites. We used the same approach to generate the chimeric antisense molecule, which is composed of the \(Fhc\)-AS fragment containing a 72-bp extension as compared with the \(Fhc\)-S fragment (spac between the sense and antisense chimeric sequences) followed by the \(Fhm\)-AS fragment (458 bp) and flanked by the HindIII and \(Xhol\) (5’-end) and BamHI (3’-end) restriction sites. The HindIII/BamHI-digested chimeric antisense fragment was inserted in the HindIII and BamHI restriction sites of the pLew100 plasmid. Then, the HindIII/Xhol-digested chimeric sense fragment was inserted, upstream of the chimeric antisense sequence, in the HindIII and \(Xhol\) restriction sites of the recombinant pLew100 plasmid. The HindIII/BamHI-digested chimeric sense/antisense cassette, extracted from the recombinant

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pLew100 plasmid, was inserted in HindIII and BamHI restriction sites of the p2T717 vector (kindly provided by B. Wickstead and K. Gull) (40).

The resulting plasmids (pLew-FHc-SAS, pLew-FHm-SAS, pLew-FHc/m-SAS, and p2T717-FHC/m-SAS containing the chimeric construct) have a sense and antisense version of the targeted gene fragment(s), separated by a 40- or 72-bp fragment, under the control of the PARP promoter linked to a prokaryotic tetracycline operator (pLew100) or under the control of two opposing tetracycline-inducible T7 promoters (p2T717-177). The EATRO1125 procyclic form cell line (EATRO1125.T7T), constitutively expressing the T7 RNA polymerase gene and the tetracycline repressor under the dependence of a T7 RNA polymerase promoter for inducible control by tetracycline (38), was transfected with the plasmids. Trypanosome transfection and selection of phleomycin-resistant clones were performed as previously reported (41).

Expression of EGFP-tagged FH in Trypanosomes—The pLew79 expression vector was used to produce recombinant FH proteins tagged with the enhanced green fluorescent protein (EGFP) optimized for fluorescence and expression in mammalian cells. The EGFP gene, preceded by a multicloning site (HindIII, BclI, XhoI, NdeI, HpaI, MluI, XbaI, and NdeI) (32) or followed by a multicloning site (XhoI, NdeI, HpaI, MluI, XbaI, and Agel) was inserted between the HindIII and BamHI restriction sites of the pLew79 vector, to generate the pLew-EGFP1 and pLew-EGFP2 plasmids, respectively. Then, PCR fragments corresponding to the first 390 amino acids of FHm (pLew-FHm-1/390.EGFP), the same region deleted for the first 333 amino acids of FHc (pLew-FHc-1/333.EGFP) were inserted into the Xhol (or HindIII) and XbaI restriction sites of the pLew-EGFP1 plasmid. In addition, a PCR fragment corresponding to the last 282 amino acids of FHc (pLew-FHc-SAS, pLew-FHm-SAS, pLew-FHc/m-SAS) was inserted between the HindIII and BamHI restriction sites of the pLew79 vector, to generate the pLew-FHc-SAS, pLew-FHm-SAS, and pLew-FHc/m-SAS plasmids, respectively. Alternatively, for ECL(TM) Western blotting detection, primary antibodies were 10 times more diluted, goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad), respectively. Alternatively, for ECL(TM) Western blotting Detection Reagents as described by the manufacturer (Amersham Biosciences). For quantitative analyses, membranes or x-ray films were scanned and protein bands were quantified using NIH Image software.

Immunofluorescence Analyses—Log phase cells were fixed with formaldehyde as described (41). Slides were incubated with rabbit anti-FHm or mouse anti-FHc antibodies (diluted 1:200) and/or monoclonal mouse anti-HSP60 (undiluted) (44) followed by Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (diluted 1:100) (Molecular Probes) and/or fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (diluted 1:100) (Bio-Rad), depending on the analyzed cell. Cells were viewed with a Zeiss UV microscope and images were captured by a MicroMax-1300Y/HS camera (Princeton Instruments) and MetaView software (Universal Imaging Corporation) and merged in Adobe Photoshop on a Macintosh iMac computer.

Enzymatic 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 fumarase activity, as previously described (45).

Expression of EGFP-tagged FH in Trypanosomes—The pLew79 expression vector was used to produce recombinant FH proteins tagged with the enhanced green fluorescent protein (EGFP) optimized for fluorescence and expression in mammalian cells. The EGFP gene, preceded by a multicloning site (HindIII, BclI, XhoI, NdeI, HpaI, MluI, XbaI, and NdeI) (32) or followed by a multicloning site (XhoI, NdeI, HpaI, MluI, XbaI, and Agel) was inserted between the HindIII and BamHI restriction sites of the pLew79 vector, to generate the pLew-EGFP1 and pLew-EGFP2 plasmids, respectively. Then, PCR fragments corresponding to the first 390 amino acids of FHm (pLew-FHm-1/390.EGFP), the same region deleted for the first 333 amino acids of FHc (pLew-FHc-1/333.EGFP) were inserted into the Xhol (or HindIII) and XbaI restriction sites of the pLew-EGFP1 plasmid. In addition, a PCR fragment corresponding to the last 282 amino acids of FHc (pLew-FHc-SAS, pLew-FHm-SAS, pLew-FHc/m-SAS) was inserted between the HindIII and BamHI restriction sites of the pLew79 vector, to generate the pLew-FHc-SAS, pLew-FHm-SAS, and pLew-FHc/m-SAS plasmids, respectively. Alternatively, for ECL(TM) Western blotting detection, primary antibodies were 10 times more diluted, goat anti-FHm or mouse anti-FHc antibodies (diluted 1:200) and/or monoclonal mouse anti-HSP60 (undiluted) (44) followed by Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (diluted 1:100) (Molecular Probes) and/or fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (diluted 1:100) (Bio-Rad), depending on the analyzed cell. Cells were viewed with a Zeiss UV microscope and images were captured by a MicroMax-1300Y/HS camera (Princeton Instruments) and MetaView software (Universal Imaging Corporation) and merged in Adobe Photoshop on a Macintosh iMac computer.

Production of FH Antibodies—Recombinant fragments, corresponding to the first 400 amino acids of FHc or FHm preceeded by a N-terminal histidine tag (6 histidine codons were introduced in the 5′ primer), were expressed in the E. coli BL21, using the pET3a expression vector (Novagen). Cells were harvested by centrifugation, and recombinant proteins were purified by nickel chelation chromatography (Novagen) according to the manufacturer’s instructions. Antisera were raised in rabbits or mice by three injections at 15-day intervals of 200 or 50 mg, respectively, of FHc-His or FHm-His recombinant nickel-purified proteins, electroeluted after separation on SDS-PAGE, and emulsified with complete (first injection) or incomplete Freund’s adjuvant. The specificity of the produced immune sera was assayed against both FH recombinant proteins expressed in E. coli, by Western blot analysis (data not shown).

Western Blot Analyses—Total protein extracts of wild type or mutant procyclic form or bloodstream form T. brucei (40°) cells were size fractionated by SDS-PAGE (10%) and immunoblotted on Immobilon-P filters (Millipore) (42). Immunodetection was performed as described (42, 43) using as primary antibodies, the monoclonal mouse anti-pyruvate phosphate dikinase H112 (undiluted) (41), the rabbit anti-FRDg (diluted 1:100) (30), the mouse anti-FHc (diluted 1:100), or the mouse anti-FHm (diluted 1:50), and as secondary antibodies, anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), respectively. Alternatively, for ECL(TM) Western blotting detection, primary antibodies were 10 times more diluted, goat anti-mouse IgG/peroxidase (1:10,000 dilution) was used as secondary antibody and revelation was done with ECL(TM) Western blotting Detection Reagents as described by the manufacturer (Amersham Biosciences). For quantitative analyses, membranes or x-ray films were scanned and protein bands were quantified using NIH Image software.

Enzymatic 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 fumarase activity, as previously described (45).

D-Glucose and Metabolite Measurements—To determine the rate of D-glucose consumption, 10⁶ procytic cells (exponential phase) were collected by centrifugation, washed in PBS, and resuspended in 10 ml of fresh SDM79 medium containing 10% calf serum. The cells were incubated for 8 h and samples (100 μl) were collected at 30-min intervals. Each sample was centrifuged at 14,000 × g for 30 s and the quantity of D-glucose remaining in the supernatant was determined using the “Glucose GOD-PAP” (Bioloab SA) kit.

To determine intracellular amounts of malate and fumarate, cell pellets (3–4 × 10⁸ cells) were washed in cold PBS and frozen in liquid nitrogen. Lysis and deproteinization of the cellular pellets involved homogenization in 500 μl of cold perchloric acid (0.9 M) and neutralization (pH 6.5) by addition of KOH/MOPS (2/0.5 M). After centrifugation, the concentrations of malate and fumarate were determined in the supernatant, by enzymatic conversion of malate and hydrazine into oxaloacetate hydrazone, with the reduction of stochiometric amounts of acetyl-pyridine-adenine dinucleotide (APAD) into APADH, which is detected at 360 nm, as described (46). The assay starts by malate determination after addition of malate dehydrogenase, and then the amount of fumarate was determined by addition of fumarase into the same cuvette.

Nuclear Magnetic Resonance Experiments—4 × 10⁸ T. brucei procyclic cells were collected by centrifugation at 1,400 × g for 10 min, washed once in PBS buffer, and incubated in 10 ml of incubation buffer (PBS buffer supplemented with 24 mM NaHCO₃ pH 7.3) containing 110 nmol of [2-13C]glucose (11
mM) for 90–180 min at 27 °C. The \( \text{d-glucose} \) concentration in the medium was determined with the "glucose GOD-PAP" (Biolabo SA) kit. The integrity of the cells during incubation was checked by microscopic observation. After centrifugation for 10 min at 1,400 \( \times \) g, the supernatant was lyophilized, re-dissolved in 485 \( \mu \text{L} \) of \( \text{D}_2\text{O} \), and 15 \( \mu \text{L} \) of pure dioxane was added as an external reference. \( ^{13}\text{C} \) NMR spectra were collected at 125.77 MHz with a Bruker DPX500 spectrometer equipped with a 5-mm broad-band probe head. Measurements were recorded at 25 °C under a bi-level broad-band gated proton decoupling with \( \text{D}_2\text{O} \) lock. Acquisition conditions were: 90° flip angle, 22,150 Hz spectral width, 64 K memory size, and 21.5 s total recycle time. Measurements were performed overnight with 2,048 scans. Spectra were obtained after a 1 Hz exponential line broadening. The recycle time (21.5 s) was too short to obtain complete relaxation of the acetate carboxyl carbon (C-1). Indeed, the C-2 signal of non-enriched acetate is 3.0-fold higher than the C-1 signal. For quantitative analyses, the C-1 values of acetate were, therefore, corrected by the 3.0 factor.

**FIGURE 1. Phylogenetic analysis of fumarases.** The phylogeny presented is based on the alignment of the entire amino acid sequences of class I and class II fumarases. The consensus tree was rooted on the class II fumarases. The branch between both class I and class II fumarases is drawn interrupted, because both fumarase classes are very distantly related. Numbers beside each node indicate bootstrap values as a percentage of 100 replicates. The species name of each sequence is indicated and the numbers in parentheses refer to the accession numbers indicated under "Experimental Procedures." The capital letters on the right indicate prokaryotes (P) and eukaryotes (E).


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FIGURE 2. Expression of the FH isoforms. In panel A, lysates (2 × 10^7 cells) of T. brucei EATRO1125.T7T procyclic (P) and AntAt1 bloodstream forms (B) were analyzed by Western blotting with the immune sera indicated below each blot. The same samples were analyzed with anti-glyceraldehyde-3-phosphate dehydrogenase (glyceraldehyde-3-phosphate dehydrogenase serving as a constitutively expressed control in each blot. The same samples were analyzed with anti-glyceraldehyde-3-phosphate dehydrogenase below each blot. The same samples were analyzed with anti-glyceraldehyde-3-phosphate dehydrogenase below each blot. The same samples were analyzed with anti-glyceraldehyde-3-phosphate dehydrogenase below each blot.

RESULTS

Characterization of Two Fumarases Expressed in Procyclic Trypanosomes—BLASTP and TBLASTN searches with fumarases from various organisms revealed two genes in the T. brucei genome, located on chromosomes 3 (Tb927.3.4500) and 11 (Tb11.02.2700). Orthologous genes are present in the genome of the related trypanosomatids, L. major (2 genes) and T. cruzi (4 genes). The identification of four related genes in T. cruzi is due to the particular structure of its genome, which is composed of two distinct haplotypes (13). A phylogenetic analysis of class I and class II fumarases shows that the trypanosomatid enzymes belong to the class I fumarases (Fig. 1). Class I fumarases are primarily found in prokaryotes and also in ancient eukaryotes of the protozoa subregnum (trypanosomatids, Plasmodium, Theileria, and Entamoeba), whereas all the other eukaryotes analyzed so far, except schistosomes, express class II fumarases.

Expression of both FH isoforms in two of the developmental stages of T. brucei was assessed by Western blot, using immune sera specific for the Tb927.3.4500 or Tb11.02.2700 gene products, called FHc and FHm, respectively. The anti-FHc and anti-FHm immune sera each recognized a single protein of 62 and 58 kDa, respectively (Fig. 2A), in agreement with the calculated sizes of the FHc and FHm gene products (62.2 and 61.3 kDa, respectively). FHc and FHm are expressed in the procyclic form, whereas neither of the FH isoforms are detectable in the bloodstream form of the parasite (Fig. 2A).

Subcellular Localization of FHm and FHc—In the procyclic form of T. brucei, fumarases convert malate into fumarate, the later being the substrate of the glycosomal and mitochondrial fumarate reductases (FRDg and FRDm1) to produce the excreted succinate (30, 32). Consequently, it is tempting to propose that these two T. brucei fumarase isoforms are glycosomal and mitochondrial enzymes. The N-terminal extremity of FHm was predicted to contain a mitochondrial targeting sequence by the MitoProt II 1.0a4 program (47) (probability 0.90), suggesting that FHm is a mitochondrial protein. The subcellular localization of FHm was studied by immunofluorescence microscopy using the specific anti-FHm immune serum (Fig. 3A). We observed a “mitochondrion-like” reticulate pattern, which was identical to the one obtained with monoclonal antibodies specific for heat shock protein HSP60, a mitochondrial marker protein (44). The mitochondrial localization was confirmed by a cellular fractionation experiment wherein the different membranes of the procyclic trypanosomes were differentially permeabilized by increasing concentrations of the detergent digitonin. Western blot analysis of the soluble fractions indicates that FHm and different mitochondrial markers (HSP60 (44), FRDm1 (32), ASCT (28), and TRYP2 (48)) are released at 350 μg of digitonin per mg of protein (data not shown). To further confirm the mitochondrial localization of FHm, we expressed in procyclic trypanosomes a fusion protein composed of the first 390 amino acids of FHm followed by EGFP. This EGFP-tagged FHm, detected by direct fluorescence analysis, shows a mitochondrial-like pattern that colocalizes with the mitochondrial HSP60 marker (Fig. 3B). The same pattern was also observed with recombinant FHm tagged with the Ty1 epitope (49) (data not shown). Moreover, a truncated form of the EGFP-tagged FHm recombinant protein that lacks the 12 N-terminal residues relocalized to the cytoplasm of the procyclic trypanosomes (Fig. 3C). These results indicate that the predicted N-terminal signal motif of FHm is required for targeting of this protein to the mitochondrion. It is noteworthy that the difference between the calculated and observed molecular masses of FHm (61.3 versus 58 kDa) probably results from the processing of its N-terminal mitochondrial-targeting motif.

Immunofluorescence microscopical analysis of the T. brucei procyclic form using the anti-FHc immune serum showed a homogenous diffuse pattern characteristic for a cytoplasmic localization (Fig. 3D). Similarly, EGFP-tagged FHc recombinant proteins composed of the first 333 amino acids (EGFP at the C-terminal end) or the last 282 amino acids (EGFP at the N-terminal end) showed the same “cytoplasmic-like” pattern (Fig. 3, E–F). This unexpected subcellular localization was confirmed by a digitonin titration experiment. Indeed, the analysis of the soluble fractions indicates that FHc and cytosolic markers (pyruvate kinase and phosphoglycerate kinase (30)) are released at 60 μg of digitonin per mg of protein, whereas the glycosomal markers pyruvate phosphate dikinase and FRDg (30, 41) are released at much higher digitonin concentrations (250 μg of digitonin per mg of protein) (data not shown).
We cannot rule out from these experiments that a small fraction of FHc or FHm is still located in glycosomes. To address this question, the presence of FHc and FHm was examined by Western blot analysis of sucrose gradient-purified glycosomes. None of the FH isoforms were detectable in the glycosomal fraction, whereas the glycosomal FRDg was enriched severalfold in this fraction (Fig. 2B), as previously described (30). The absence of fumarase in T. brucei glycosomes is strengthened by the absence of recognizable PTS1 or PTS2 glycosomal targeting motifs in both FHc and FHm sequences.

**FHc and FHm Account for the Cellular Fumarase Activity**—We generated separate mutant procyclic cell lines for RNAi-mediated repression of FHc, FHm, and both simultaneously (15), similarly as previously described for the FRD isoforms (30, 32). The pLew100 vector (39) was used to produce from a single tetracyclin-inducible promoter the hairpin double-stranded RNA molecules, as described (25, 28, 30, 32, 38). The recombinant pLew100-derived plasmids were introduced into the EATRO1125.T7T cell line expressing the tetracycline repressor (38, 39).

We have selected two ΔFHc mutant cell lines (ΔFHc-A5, ΔFHc-D4), one ΔFHm mutant cell line (ΔFHm-A1), and four double mutant cell lines inhibited for the expression of both FHc and FHm isoforms (including ΔFHc/m-B5). In the absence of tetracycline (ni), the expression of the hairpin double-stranded RNA is inhibited by the tetracycline repressor, which binds to its operator located between the transcription promoter and the RNAi cassette. Nevertheless, fumarase activity in the ΔFHm-A1.ni (Fig. 4A), ΔFHc-A5.ni (Fig. 4B), ΔFHc-D4.ni (data not shown), and ΔFHc/m-B5.ni (Fig. 4C) noninduced cell lines is 1.3–2-fold reduced, probably due to leakage of the inducible expression system, as previously observed (25, 28, 30, 32, 38, 39). After 7 days of tetracycline induction (i), the protein encoded by the RNAi-repressed gene was not detectable anymore by Western blot analysis, serving as loading and specificity control, as not affected. Upon repression of the cytosolic (FHc) or mitochondrial (FHm) fumarases, total cellu-
lar fumarase activity was 3- or 2.5-fold reduced, respectively, indicating that the activity is slightly higher in the cytosolic compartment compared with the mitochondrion (Fig. 4, A and B). However, inactivation of both FHc and FHm expression was still incomplete 7 days post-induction of the ΔFHc/m-B5.i double mutant, because 16.6% of the fumarase activity remained and both FH isoforms are detectable by Western blot analysis (Fig. 4C and Table 1). The residual expression of both FHc and FHm in the ΔFHc/m-B5.i double mutant, whereas both proteins were undetectable in all the single mutant cell lines analyzed (Fig. 4 and Table 1), suggests that fumarase may be essential for cell viability. Indeed, among the three other double mutant cell lines obtained, one died after a few days of culturing in the absence of tetracycline (probably because of a high level of repression leakage) and two others rapidly reverted 3–4 days post-tetracycline induction, by re-expressing the targeted FHc and FHm genes (data not shown).

To prevent the presumed negative effect of repression leakage, we used an alternative expression vector (p2T7T1-177) designed for integration in the transcriptionally silent minichromosomes (40). In addition, the mRNA target sequence is flanked by two opposing tetracycline-inducible T7 promoters, which considerably increase the level of hairpin double-stranded RNA expression compared with the single promoter pLew100 vector.4 We have selected two double mutant clones, one showing a residual fumarase activity upon tetracycline induction (ΔFHc/m-E9.i) (Table 1), whereas in the

4 L. Rivière and F. Bringaud, unpublished data.
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Effect of FH depletion on the doubling time and glucose consumption of procyclic T. brucei cell lines

| Cell line          | Vector<sup>a</sup> | FH activity | Protein<sup>b</sup> | Doubling time | Glucose consumption |
|--------------------|--------------------|-------------|--------------------|---------------|---------------------|
|                    |                    | milliunits g<sup>-1</sup> of protein | %       | h               | µmol h<sup>-1</sup> mg<sup>-1</sup> of protein |
| EATRO1125.T7T      |                    | 307 ± 13    | 100                | 13.5 ± 0.9    | 1.66 ± 0.06         |
| ΔFRDg/m1           | B5.ni<sup>c</sup>  | 53.8        | ND                 | 13.0 ± 1.1    | 1.12 ± 0.08         |
| ΔFRDg/m1           | B5.j<sup>c</sup>   | 53.8        | ND                 | 13.0 ± 1.1    | 1.12 ± 0.08         |
| ΔFHm               | A1.ni              | 192 ± 8     | 42.9               | 22.3 ± 1.5    | 1.07 ± 0.02         |
| ΔFHm               | A1.i               | 122 ± 2     | ND                 | 23.1 ± 1.6    | 1.01 ± 0.02         |
| ΔFHc               | A5.ni              | 165 ± 6     | 27.4               | 19.4 ± 1.3    | 1.05 ± 0.04         |
| ΔFHc               | A5.i               | 98 ± 5      | ND                 | 43.6 ± 1.3    | 0.83 ± 0.13         |
| ΔFHc               | D4.ni              | 155 ± 11    | 24.8               | 19.3          | ND                  |
| ΔFHc               | D4.i               | 102 ± 13    | ND                 | 46.3          | 0.80 ± 0.05         |
| ΔFHc/m             | B5.ni              | 242 ± 26    | 55.5               | 17.7 ± 1.0    | 1.21 ± 0.08         |
| ΔFHc/m             | B5.i               | 51 ± 6      | 7.2                | 30.2 ± 1.2    | 0.85 ± 0.09         |
| ΔFHc/m             | E9.ni              | 260 ± 27    | 56.1               | 16.4          | ND                  |
| ΔFHc/m             | E9.i               | 21 ± 5      | 4.7                | 50.1 ± 1.8    | 0.69 ± 0.06         |
| ΔFHc/m             | F10.ni             | 163 ± 20    | 35.3               | 19.7          | 1.01 ± 0.07         |
| ΔFHc/m             | F10.i              | 107 ± 18    | 13.5               | 12.1 ± 0.9    | 0.84 ± 0.08         |

<sup>a</sup> Vector used for double-stranded RNA expression (RNAi).
<sup>b</sup> Relative amounts of the targeted protein(s) in the mutant expressed as a percentage of the protein(s) detected by Western blotting in the parental cell line.
<sup>c</sup> Data from Ref. 32.
<sup>d</sup> ND, not determined.
<sup>e</sup> Not viable.

FIGURE 5. Effect of inactivation of the FH genes expression and addition of extracellular fumarate on the ΔFHc/m-F10 mutant viability. The cumulative cell density of the non-induced (filled square) and tetracycline-induced (circle) ΔFHc/m-F10 mutant cell line grown in SDM79 was estimated every day to determine the doubling time, which is indicated at the top of each curve when the cells are viable. To study the effect of fumarate, tetracycline was added at the beginning of the experiment (open circle) followed by the addition of 10 mM fumarate 7 days after induction (filled circle).

ΔFHc/m-F10.i cell line both the fumarase activity and the FH proteins are not detectable (Fig. 4D and Table 1). This clearly demonstrated that FHc and FHm together account for all cellular fumarase activity.

Effect of FH depletion on the survival of procyclic T. brucei—Fumarases are essential for cell viability, because the ΔFHc/m-F10.i cell line dies 12 days after tetracycline induction (growth arrest is observed 6 days post-induction), when the standard procedure is used to maintain the cell culture (dilution at 1 × 10<sup>7</sup> cell ml<sup>-1</sup>) (Fig. 5). It is noteworthy that this cell line can be artifically maintained by diluting stepwise the culture every 2 days until a significant re-expressing of the targeted FHc and FHm genes occurs (data not shown). The ΔFHc/m-B5.i and the ΔFHc/m-E9.i double mutant cell lines are viable, probably because of the incomplete inhibition of FH expression (Table 1). However, the ΔFHc/m-E9.i cell line shows a considerable increase of its doubling time (3.6-times compared with the parental cell line), a long “nasal” shape and a reduced motility (the cells lie on the bottom of the flask). Consequently, this cell line is too fragile for biochemical analyses in PBS buffer, as also observed the ΔFHc/m-F10.i cell line.

The doubling time of all analyzed tetracycline-induced single mutant cell lines also increased (23.1–46.3 versus 13.5 h for the parental cells), indicating that both fumarases play an important role in the intermediary metabolism of these parasites (Table 1). The ΔFHc mutants are more affected than the ΔFHm mutant (46.3 and 43.6 versus 23.1 h for ΔFHc-A5.i, ΔFHc-D4.i, and ΔFHm-A1.i, respectively). In addition, the motility of the ΔFHc-A5.i and ΔFHc-D4.i mutants is reduced, although not as much as that of the ΔFHc/m-E9.i cell line. Incidentally, the growth rate of the non-induced cell lines is also affected (Table 1), probably as a consequence of the presumed repression leakage.

Effect of FH Depletions on Glucose Metabolism—The rate of glucose consumption of the wild type cell line and all six mutant cell lines (tetracycline-induced and non-induced) was measured during 8 h in SDM79 medium, as previously described (32). The non-induced and tetracycline-induced mutant cell lines all showed a reduced rate of glucose consumption (from 0.69 to 1.21 mmol h<sup>-1</sup> mg<sup>-1</sup> of protein) compared with the parental EATRO1125.T7T cell line (1.66 mmol h<sup>-1</sup> mg<sup>-1</sup> of protein) (Table 1). The lowest rates, observed for the ΔFHc/m-B5.i and ΔFHc/m-E9.i double mutants, are similar to the rate previously reported for the ΔFRDg/m1-B5.i mutant cell line depleted for both the glycosomal and the mitochondrial FRD (0.77 mmol h<sup>-1</sup> mg<sup>-1</sup> of protein) (32).

To further explore the impact of fumarase depletion on glucose catabolism, we performed an NMR analysis of 13C-enriched end products excreted by untransfected and mutant cell lines (ΔFHc-A5, ΔFHm-A1, and ΔFHc/m-B5) incubated in the presence of d-[1-13C]glucose, as the only carbon source (Table 2). The EATRO1125.T7T procyclic cell line mainly excretes succinate, acetate, and lactate (63.3, 25, and 7.4% of the excreted 13C-enriched molecules, respectively) and small amounts of malate, fumarate, and alanine (2.6, 0.5, and 1.1%, respectively).
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TABLE 2
Excreted end products of D-[1-13C]glucose metabolism by procyclic T. brucei cell lines

| Cell line          | FH activity | Nanomole of 13C-enriched excreted molecules* | h⁻¹ mg⁻¹ of protein |
|--------------------|-------------|---------------------------------------------|--------------------|
| EATRO1125.T7T      | 307 ± 13    | Succinate | Acetate | Lactate | Malate | Fumarate | Alanine | Glycerol | Total |
| ΔFRDg/m1          | B5.i        | 372 ± 27  | 143 ± 13 | 53 ± 11 | 24 ± 5.8 | 18 ± 1.9 | 18 ± 1.0 | 10 ± 1.1 | 7 ± 0.7 |
| ΔFHm              | A1.i        | 255 ± 28  | 147 ± 11 | 10 ± 1.1 | 10 ± 1.3 | 7 ± 0.7 | 5 ± 0.4 | 2 ± 0.9 | 7 ± 4.1 |
| ΔFHc              | A5.i        | 232 ± 26  | 180 ± 6  | 30 ± 10 | 22 ± 6.6 | 3 ± 0.2 | 13 ± 5.0 | ND | ND |
| ΔFHc/m            | B5.i        | 217 ± 11  | 99 ± 7   | 2 ± 1   | 132 ± 21 | 2 ± 0.3 | 16 ± 7.2 | 480 ± 77 | 7 ± 3.3 |

% of 13C-enriched excreted molecules

| EATRO1125.T7T      | 307 ± 13    | 63.3 ± 2.5 | 74 | 2.6 | 0.5 | 1.1 | ND |
| ΔFRDg/m1          | B5.i        | 61.0 ± 2.6 | 7.5 | 2.6 | 0.4 | 1.5 | 0.2 |
| ΔFHm              | A1.i        | 58.7 ± 2.5 | 9.2 | 4.3 | 0.5 | 1.4 | 0.1 |
| ΔFHc              | A5.i        | 47.3 ± 2.7 | 3.9 | 5.0 | 0.2 | 3.1 | ND |
| ΔFHc/m            | B5.i        | 64.5 ± 2.3 | 6.6 | 4.0 | 0.2 | 1.7 | ND |
| ΔFHc/m            | B5.i        | 45.1 ± 2.0 | 0.3 | 23.7 | 0.2 | 1.3 | 2.5 |

* The values for the EATRO1125.T7T and ΔFRDg/m1-B5.i cell lines are slightly different from previous publications (30, 32), because the [1-13C]glucose was considered in the present analysis.

As expected, a reduction of succinate excretion in the mutant cell line, which shows approximately one-third of the wild type activity (30%), as compared with the parental EATRO1125.T7T cell line (51–58%). Interestingly, the ΔFHc/m-B5.i cell line excreted 7.2-fold more [3-13C]malate than [2-13C]malate (Table 4). This suggests that the reaction catalyzed by fumarases is no longer reversible in the ΔFHc/m-B5.i mutant cell line, probably because the enzymes have become rate-limiting after a 6-fold reduction of their activities. In contrast, the [3-13C]malate/[2-13C]malate ratio of the single mutant cell lines, which show approximately one-third of the wild type fumarase activity (98–122 versus 307 mmol h⁻¹ mg⁻¹ of protein), respectively, with a total of 743 nmol of 13C-enriched excreted molecules per h and per milligram of cellular proteins. (Fig. 6A, Table 2). We observed a 1.3–1.5-fold reduction of the rate of the 13C-enriched molecules excretion in tetracycline-induced mutant cell lines, as compared with the wild type cell line (Table 2), which is in agreement with the observed reduction of the D-glucose consumption rate (Table 1). Inhibition of mitochondrial or cytosolic fumarase expression induces a 7 (ΔFHm-A1.i) or 23% (ΔFHc-A5.i) reduction of the relative excretion of 13C-enriched succinate, respectively, as compared with the parental EATRO1125.T7T cell line (58.7 and 47.3% versus 63.3% of the 13C-enriched excreted molecules, respectively). The relatively low level of inhibition of succinate excretion (or increase of malate expression), although FHc and FHm are undetectable in the ΔFHc-A5.i and ΔFHm-A1.i single mutant cell lines, respectively, suggests that the flux is redirected to the non-targeted isoform, as previously observed for the ΔFRDg and ΔFRDm1 mutant cell lines (32). This implies that both fumarase isoforms are involved in succinate production pathways, a notion that is strengthened by the significant reduction of succinate production (30% as compared with the wild type cells), caused by the 6-fold reduced fumarase activity in the ΔFHc/m-B5.i double mutant cell line (51 versus 307 μmol h⁻¹ mg⁻¹ of protein).

As expected, a reduction of succinate excretion in the mutant cell lines is correlated to an increase of malate production, the substrate of fumarases. When 63.3, 58.7, 47.3, and 45.1% of the end products are succinate, malate represents 2.6, 4.3, 18.8, and 27.5% of the excreted end products, in the wild type, ΔFHm-A1.i, ΔFHc-A5.i, and ΔFHc/m-B5.i cell lines, respectively. Surprisingly, the amounts of excreted 13C-enriched fumarate, the product of the reaction catalyzed by fumarases, are not significantly reduced in the mutant cell lines, even in the ΔFHc/m-B5.i cell line that shows a 9.8-fold increase in the relative amount of malate excretion (Table 2). Because, the amounts of excreted fumarate are very low and thus may not reflect exactly the intracellular situation, we determined the 13C-enriched molecules in cell pellets collected from the previous NMR experiments. Unfortunately, the NMR approach appeared not sensitive enough to detect malate and fumarate in the cell pellets, whereas accumulation of succinate, alanine, and aspartate inside the cells could be measured (data not shown). Alternatively, we used an enzymatic method to determine the intracellular amounts of fumarate and malate in cells grown in the SDM79 medium (Table 3). The parental cell line contains 0.8 nmol of fumarate per mg of protein and 1.7-fold more malate. As expected, both the fumarate and malate amounts considerably increased in the ΔFRDg/m1-B5.i mutant (5.3- and 6.6-fold, respectively). Upon silencing of fumarase genes, intracellular amounts of fumarate decreased, whereas intracellular amounts of malate increased. A correlation is observed between the fumarate decrease and the malate increase, which is expected in fumarase mutants (Fig. 7). Interestingly, modification of the intracellular amounts of fumarate and/or malate is related to the doubling time (Fig. 7).
tein), only slightly increased as compared with the parental cell line (1.3–1.7 versus 1.1) (Table 4). A 3-fold reduction of the cellular fumarase activity does not significantly affect the reversibility of the reaction, which implies that the fumarase activity is in excess and thus not rate-limiting for succinate production.

This apparent loss of reversibility is also in the labeling of metabolites further upstream in the pathway, because the [3-13C]/[2-13C]alanine, [3-13C]/[2-13C]lactate, and [2-13C]/[1-13C]acetate ratios also considerably increased in the ∆FHc/m-B5.i mutant compared with the parental and ∆FRDg/m1-B5.i cell lines (21.9 versus 2.9–3.2, 6.4 versus 1.8–3.2 and 8.2 versus 2.3–2.7, respectively) (Table 4). This indicates that the PEP carboxykinase and malate dehydrogenase steps (steps 12 and 13 in Fig 8) are also reversible in wild type procyclic cells, although one cannot exclude that the malic enzyme step (step 16), which is considered as negligible under standard growth conditions, is also involved in production of [2-13C]alanine, [2-13C]lactate, and [1-13C]acetate (Fig 8).

Fumarate Rescues FH Depletions—To determine whether reduction of fumarate production (or increase of the intracellular malate/fumarate ratio) is responsible for the growth phenotype, the wild type and mutant (non-induced and tetracycline-induced) cell lines were incubated with fumarate. Table 3 shows the doubling time, the rate of glucose consumption, and the intracellular amounts of malate and fumarate in cell lines incubated in the presence of increasing concentrations of fumarate. The doubling time of wild type cells is not affected by addition of up to 1 mM fumarate to the growth medium. Addition of 2–20 mM fumarate, however, slightly affects the doubling time (14.3–15.3 versus 13.5 h), the effect is more pronounced in the presence of 50 mM fumarate (17.9 h) and 100 mM fumarate is lethal for the parasite. The doubling time of the non-induced ∆FHc-A5.ni and ∆FHc-D4.ni mutant cell lines is significantly shortened in the presence of 10 mM fumarate (17.4 and 17.3 versus 19.4 and 19.3 h, respectively), to reach a value close to that of wild type cells grown under the same conditions (15.3 h). Important changes were observed for the tetracycline-induced ∆FHc-A5.i and ∆FHc-D4.i cell lines, which grow ~2 times faster in the presence of 20 mM fumarate. Equivalent data were obtained for the other non-induced and tetracycline-induced ∆FH mutant cell lines analyzed (Table 3). More interesting is the partial rescue observed for the lethal ∆FHc/m-F10.i mutant grown in the presence of 10 mM fumarate (Fig 5), which clearly demonstrates that production of fumarate by procyclic trypanosomes is necessary for cell viability.

As expected, addition of fumarate to the growth medium causes an increase of the intracellular amounts of fumarate in all cell lines analyzed, including wild type cells (Table 3). The ∆FHm-A1.i and wild type cell lines showed a moderate increase (up to 0.9–1.2 nmol mg⁻¹ of protein, in the presence of 20 mm were obtained after addition of 15 µl of dioxane (D). Each spectrum corresponds to one representative experiment from a set of at least 3. The resonances were assigned as follows: A, acetate; AI, L-alanine; D, dioxane; F, fumarate; G, D-glucose; GI, glycerol; L, lactate; M, malate; S, succinate. The position of the enriched 13C in each detected molecule is indicated by its number in subscript. For succinate and fumarate C-2 and C-3 resonances (C-1 and C-3 for glycerol) are undistinguishable and were labeled “2,3” (“1,3” for glycerol).
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TABLE 3
Effect of fumarate addition to the growth medium of procyclic T. brucei cell lines

| Cell line | [Fumarate]$^a$ | Malate | Fumarate | Ratio$^b$ | Doubling time | Glucose consumption |
|-----------|----------------|--------|----------|-----------|---------------|---------------------|
|           | mmol mg$^{-1}$ of protein | h | mmol h$^{-1}$ mg$^{-1}$ of protein |           |               |                     |
| EATRO1125:T7T | 0  | 1.36 ± 0.18  | 0.80 ± 0.03  | 1.70 | 13.5 | 1.66 ± 0.06   |
|           | 2  | 1.59 ± 0.08  | 0.95 ± 0.07  | 1.68 | 14.3 | ND$^d$         |
|           | 5  | 1.67 ± 0.11  | 1.11 ± 0.28  | 1.50 | 14.3 | ND              |
|           | 10 | 1.84 ± 0.15  | 1.17 ± 0.20  | 1.57 | 15.3 | 1.59 ± 0.08   |
|           | 20 | 1.97 ± 0.20  | 1.22 ± 0.17  | 1.62 | 15.3 | ND              |
|           | 50 | ND           | ND          | ND   | 17.9 | ND              |
| ∆FRDg/m1  | B5.i | 0  | 8.96 ± 0.26  | 4.23 ± 0.25  | 2.12 | 13.4 | 0.77 ± 0.04   |
| ∆FHm      | A1.ii | 0   | 1.72 ± 0.23  | 0.73 ± 0.01  | 2.35 | 22.3 | 1.07 ± 0.02   |
|           | 10  | 1.86 ± 0.28  | 0.93 ± 0.29  | 1.99 | 19.7 | 1.12 ± 0.04   |
|           | 20  | 2.06 ± 0.17  | 1.10 ± 0.13  | 1.88 | 19.4 | 1.17 ± 0.03   |
|           | 50  | 2.14 ± 0.30  | 1.15 ± 0.13  | 1.86 | 28.4 | 1.02 ± 0.04   |
| ∆FHm      | A1.i | 0  | 2.01 ± 0.04  | 0.63 ± 0.09  | 3.22 | 23.1 | 1.01 ± 0.02   |
|           | 10  | 2.49 ± 0.28  | 0.79 ± 0.11  | 3.17 | 20.6 | 1.43 ± 0.07   |
|           | 20  | 2.60 ± 0.26  | 0.87 ± 0.01  | 2.99 | 19.9 | ND              |
|           | 50  | 2.83 ± 0.19  | 1.08 ± 0.11  | 2.62 | 26.4 | 1.30 ± 0.07   |
| ∆FHc      | A5.ii | 0 | 1.84 ± 0.08  | 0.91 ± 0.01  | 2.02 | 19.3 | 1.05 ± 0.04   |
|           | 1   | 2.22 ± 0.23  | 1.23 ± 0.12  | 1.81 | 18.0 | ND              |
|           | 2   | 2.27 ± 0.13  | 1.40 ± 0.03  | 1.62 | 17.4 | 1.16 ± 0.04   |
|           | 5   | 2.89 ± 0.14  | 1.75 ± 0.26  | 1.65 | 17.9 | 1.39 ± 0.02   |
|           | 10  | 3.22 ± 0.23  | 1.86 ± 0.05  | 1.73 | 17.3 | 1.65 ± 0.14   |
|           | 20  | 3.28 ± 0.11  | 2.06 ± 0.09  | 1.59 | 17.9 | 1.11 ± 0.09   |
|           | 50  | 5.49 ± 0.21  | 3.31 ± 0.25  | 1.66 | 29.8 | 0.89 ± 0.09   |
| ∆FHc      | A5.ii | 0 | 8.70 ± 0.26  | ≤0.20 ± 0.05$^d$  | 43.52 | 46.3 | 0.83 ± 0.05   |
|           | 0.5 | 2.85 ± 0.11  | 0.49 ± 0.16  | 5.81 | 45.6 | ND              |
|           | 1   | 2.91 ± 0.07  | 0.85 ± 0.01  | 3.41 | 40.7 | ND              |
|           | 2   | 3.73 ± 0.04  | 1.18 ± 0.03  | 3.15 | 39.1 | 0.96 ± 0.10   |
|           | 5   | 4.48 ± 0.17  | 1.53 ± 0.25  | 2.94 | 34.2 | 1.05 ± 0.21   |
|           | 10  | 4.82 ± 0.24  | 1.75 ± 0.22  | 2.75 | 32.7 | 1.29 ± 0.22   |
|           | 20  | 5.69 ± 0.17  | 2.98 ± 0.14  | 1.91 | 23.9 | 1.22 ± 0.11   |
|           | 50  | 6.20 ± 0.26  | 3.14 ± 0.29  | 1.97 | 32.4 | 1.06 ± 0.01   |
| ∆FHc/m    | B5.ii | 0 | 1.52 ± 0.13  | 0.77 ± 0.1  | 1.96 | 17.7 | 1.21 ± 0.08   |
| ∆FHc/m    | B5.i | 0  | 4.36 ± 0.11  | 0.30 ± 0.10  | 14.52 | 30.2 | 0.85 ± 0.09   |
|           | 0.5 | 2.85 ± 0.27  | 0.75 ± 0.02  | 3.79 | 27.6 | ND              |
|           | 1   | 3.29 ± 0.17  | 1.08 ± 0.11  | 3.04 | 27.6 | ND              |
|           | 2   | 3.60 ± 0.21  | 1.30 ± 0.18  | 2.77 | 28.7 | 1.39 ± 0.10   |
|           | 5   | 4.36 ± 0.06  | 1.52 ± 0.09  | 2.87 | 28.7 | ND              |
|           | 10  | 6.38 ± 0.30  | 2.49 ± 0.24  | 2.56 | 27.4 | ND              |
|           | 20  | 6.46 ± 0.28  | 2.54 ± 0.10  | 2.54 | 23.7 | ND              |
|           | 50  | 6.65 ± 0.24  | 2.93 ± 0.24  | 2.27 | 25.7 | 0.61 ± 0.09   |

$^a$ Fumarate concentration (mM) in the growth medium.
$^b$ Intracellular malate/fumarate ratio.
$^c$ ND, not determined.
$^d$ This value may be underestimated, because it is close to the background value.

fumarate) as compared with the ∆FHc/m-B5.i and ∆FHc-A5.i mutants (up to 2.5–3 nmol mg$^{-1}$ of protein, in the presence of 20 mM fumarate). The same phenomenon was observed for the intracellular amounts of malate, which moderately increased in extracellular fumarate. These differences observed between cell lines seem to be related to the inhibition of FH gene expression, but the explanation remains unknown. In addition, all mutant cell lines showed a decrease in the malate/fumarate ratio, which is up to 23-fold reduced in the ∆FHc-A5.i mutant. The intracellular malate/fumarate ratio of the FH mutants is restored in the presence of 20–50 mM fumarate. Another consequence of the incubation in the presence of fumarate is the increase of glucose consumption in ∆FH mutant cell lines, to reach a value close to that of wild type cells (Table 3). It is noteworthy that the positive effect of fumarate is observed when it is added extracellularly, and in a concentration dependent manner. Altogether, these data suggest that the partial rescue of FH depletion, by addition of fumarate to the growth medium, is due to the increase of intracellular fumarate amounts, decrease of the intracellular malate/fumarate ratio, and/or increase of the rate of glucose consumption.

DISCUSSION

We previously characterized two NADH-dependent fumarate reductase isoforms involved in succinate production from glucose metabolism, a glycosomal enzyme (FRDg) and a mitochondrondial enzyme (FRDm1) (30, 32). Screening of the T. brucei genome revealed two genes similar to class I fumarase genes (FHc and FHm), which encode the enzymes catalyzing the reaction preceding the FRD step in the glucose metabolism pathways. One would have expected that the fumarases are present in the same subcellular compartments as the FRD isoforms. Indeed, FHm is located in the mitochondrion and thus probably provides fumarate to FRDm1, which reduces it into excreted succinate. Because fumarases catalyze a reversible reaction, FHm also function in the oxidative direction (tricarboxylic
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Acid cycle. However, the tricarboxylic acid enzymes are not working in this parasite as a full cycle fed by acetyl-CoA when grown in glucose-rich medium (27). In contrast, part of the tricarboxylic acid cycle, including FHm, may be used by procyclics of the TREU927 strain grown in glucose-rich medium, to convert 1-proline into acetate (50). Nonetheless, the involvement of FHm in the 1-proline metabolism is debatable, because, the only end products excreted from 1-proline by another procyclic strain (AnTat1.1 strain), grown under the same conditions, are succinate and CO$_2$ (27).

In contrast, neither FHc nor FHm are detectable in the glycosomal fraction, where FRDg is located. This data are recently confirmed by a mass spectrometry-based glycosomal proteomics analysis, which did not detect any of the FH isoforms (51). The apparent absence of glycosomal fumarase is supported by the demonstration of the cytosolic localization of FHc. We previously detected a significant fumarase activity in the glycosomes. Indeed, according to the current model, FRDg requires to reoxidize half of the NADH produced by the glycosomal glyceraldehyde-3-phosphate dehydrogenase (21, 22). Depletion of the mitochondrial FRD (FRDm1) may also affect the mitochondrial redox balance, although it is debatable, because of the presence of other mitochondrial NADH dehydrogenases linked to the respiratory chain (32, 52, 53). The analysis of ΔFH mutants, which are affected for the catalytic steps preceding the FRD reaction, provides additional information regarding the role of FRD in the procyclic trypanosomes. Indeed, addition of fumarate to the medium restores the rate of glucose consumption by the ΔFH mutants. In the presence of external fumarate, both FRD isoforms can produce succinate and thus participate in the maintenance of the redox balance. This suggests that the observed decrease in the rate of glucose consumption by the ΔFH mutants is due to the impossibility of the FRD isoforms to reoxidize NADH, because the other substrate (fumarate) is not available.

Although the T. brucei ΔFH and ΔFRD mutant cell lines show equivalent reduction of the rate of glucose consumption, cell viability is very different. Indeed, in the absence of detectable activity of the targeted enzymes, the ΔFRDg/m1 mutant cell lines behave as the wild type cells, whereas the ΔFHc/m-F10.i cell line is not viable. In addition, a partial inhibition of both FH isoforms (83.4% inhibition of the cellular fumarase activity) strongly affects the doubling time of the mutant. An important question to address is what is the reason of the growth phenotype observed for the ΔFH mutants? As mentioned above, the decrease of the glucose consumption rate observed in both the ΔFH and ΔFRD mutants does not seem to be responsible for the growth phenotype, because the doubling time of the ΔFRD mutants is not affected. The only other difference observed is related to decrease and increase of the intracellular amounts of fumarate and malate, respectively. Addition
of 10–20 mM fumarate to the growth medium restores the intracellular fumarate concentration and the malate/fumarate ratio. It also partially rescues the growth phenotype of all the analyzed mutants, including the non-viable ΔFHc/m-F10.i cell line. This indicates that fumarate is an essential intermediary metabolite produced by the procyclic trypanosomes. The partial rescue may be interpreted as a balance between positive and negative effects of fumarate, because, the wild type cells affected by over 10 mM fumarate (at 100 mM it is lethal). Recently, Annoura et al. (54) demonstrated that the dihydroorotate dehydrogenase (DHOD) from *T. cruzi*, which is a soluble protein involved in *de novo* pyrimidine biosynthesis, is essential for cell viability of the parasite (54). This enzyme also acts as a fumarate reductase, because fumarate is used as an electron acceptor instead of NAD<sup>+</sup>, as observed for other DHODs belonging to family 1A (55). Consequently, this essential enzyme depends on fumarate produced by *T. cruzi* and probably other trypanosomatids, which also synthesize pyrimidines *de novo* (56, 57) and contain a closely related DHOD gene (the *T. brucei* and *T. cruzi* DHOD are 80% identical) (data not shown). Interestingly, DHOD and one fumarase isofrom (FHc) are cytosolic enzymes (57, 58). Depletion of the *T. brucei* FHc gene induces a severe growth phenotype (the growth phenotype of FHm mutant is less severe), suggesting that the cytosolic isofrom is particularly important for trypanosome viability. Consequently, it is tempting to propose that the cytosolic location of FHc, which is unexpected because most of the corresponding succinate production pathway takes place in glycosomes, is necessary for the cytosolic production of fumarate to feed enzymes such as DHOD. This view also implies that the role of the succinate fermentation pathways in trypanosomes (at least the glycosomal/lysosomal one) is not only to maintain the redox balance in organelles (glycosomes and possibly the mitochondrion), but also to provide at least one essential metabolic enzyme with fumarate. Further experiments will be required to determine which of these two roles of the succinic pathway is the most important one and whether their importance is dependent on growth.

**TABLE 4**

| Cell line   | Malate [3-13C]/[2-13C] | Alanine [3-13C]/[2-13C] | Lactate [3-13C]/[2-13C] | Acetate [3-13C]/[2-13C] |
|-------------|------------------------|-------------------------|-------------------------|------------------------|
| EATRO112S.T7T | 1.08 ± 0.13             | 3.19 ± 1.05             | 3.17 ± 0.71             | 2.73 ± 0.44            |
| ΔFRDg/m     | 1.12 ± 0.02             | 2.88 ± 1.08             | 1.83 ± 0.32             | 2.34 ± 0.62            |
| ΔFHm        | 1.28 ± 0.19             | 2.73 ± 0.51             | 3.01 ± 0.08             | 2.73 ± 0.04            |
| ΔFHm        | 1.71 ± 0.01             | 4.65 ± 0.25             | 3.65 ± 0.19             | 3.35 ± 0.39            |
| ΔFHc        | 1.66 ± 0.04             | 3.26 ± 0.16             | 3.20 ± 0.13             | 3.22 ± 0.14            |
| ΔFHc        | 1.64 ± 0.05             | 3.26 ± 0.05             | 1.93 ± 1.06             | 2.34 ± 0.10            |
| ΔFHc/m      | 1.34 ± 0.04             | 3.02 ± 0.61             | 2.97 ± 0.04             | 2.53 ± 0.09            |
| ΔFHc/m      | 7.16 ± 1.06             | 21.86 ± 0.10            | 6.42 ± 2.44             | 8.15 ± 1.10            |

**FIGURE 8. Pathway for 13C-enriched succinate, acetate, lactate, and alanine of the procyclic *T. brucei* incubated with D-[1-13C]glucose.** This figure shows the position of 13C in glucose, intermediary metabolites, and excreted end products (boxed molecules) of D-[1-13C]glucose metabolism in procyclic trypanosomes. The name of the 13C-enriched molecules detected in the incubation medium is bold- face and underlined. Black dots with white numbers indicate 13C-enriched carbons corresponding to position C-1 of glucose. The wild type cells convert D-[1-13C]glucose into (3-13C)PEP (13C-enriched at position 3). According to the current model, approximately half of the PEP is converted into the excreted (3-13C)lac- tate, (3-13C)alanine, and (2-13C)acetate, whereas the other half is converted into (3-13C)- and (2-13C)succinate (22, 30). Because succinate and fumarate have a symmetrical structure, the same amounts of molecules are 13C-enriched at positions C-2 or C-3 (gray dots with white numbers). The detection of (2-13C)lactate, (2-13C)alanine, and (1-13C)acetate suggests that the catalytic steps producing fumarate from PEP are reversible (thin arrows in the phosphoenolpyruvate carboxykinase, malate dehydrogenase, and FH steps). Indeed, the C-2 position of PEP, which corresponds to the C-2 position of lactate and alanine and the C-1 position of acetate (white dot with black numbers), was derived from the C-2 position of malate (and fumarate). The analyzed mutant cell lines also produce detectable amounts of glycerol 13C-enriched at positions 1 and 3, because of its symmetrical structure. The dashed arrow indicates a step, which is supposed to occur at a background level or not at all. Abbreviations: DHAP, dihydroxyacetone phosphate; F1,6-bisP, fructose 1,6-bisphosphate; G3P, glycerol 3-phosphate; GAP, glyceraldehyde-3-phosphate. Enzymes: 1) hexokinase; 2) glucose-6-phosphate isomerase; 3) phosphofructokinase; 4) aldolase; 5) triose-phosphate isomerase; 6) 3-phosphoglycerate dehydrogenase; 7) glyceraldehyde-3-phosphate dehydrogenase; 8) phosphoglycerate kinase; 9) phosphoglycerate mutase; 10) enolase; 11) phosphoenolpyruvate carboxykinase; 12) malate dehydrogenase; 13) fumarase (FHc and FHm); 14) NADH-dependent fumarate reductase (FRDg and FRDm1); 15) malic enzyme; 16) pyruvate kinase; 17) pyruvate phosphokinase; 18) unknown reaction (lactate dehydrogenase has never been identified in *T. brucei*); 19) another unknown enzyme.
conditions. Incidentally, in the absence of detectable NADH-FRD activity and detectable FRD proteins (ΔFRDg/m1 cell lines), the production of succinate is reduced by 92% as compared with the wild type cells (32). DHOH and additional FRD activity(ies) may account for the remaining 8% of excreted succinate in these mutant cell lines. In light of these data, it would be interesting to study the substrate specificity of other soluble oxidoreductases in trypanosomatids, to eventually identify other enzymes with fumarate reductase activity.

In conclusion, our reverse genetic approach and the localization and metabolic studies show that the identified T. brucei FH genes code for mitochondrial and cytosolic class I fumarases, which are essential for procyclic trypanosome viability. Both cytosolic and mitochondrial isofoms expressed by the mammalian hosts belong to the class II fumarases. Because class I and class II fumarases are very distantly related enzymes, one may consider that the development of trypanosome-specific inhibitors against fumarases might produce anti-trypanosomatid drugs. Unfortunately, this strategy would not be useful against African trypanosomiasis, as FH isoforms are not detected in the bloodstream stage of T. brucei infecting the mammalian host. These enzymes are, however, probably present in human-infective stages of other trypanosomatids including Leishmania and T. cruzi, which also excrete succinate from glucose metabolism (19). Class I fumarase genes are also present in the genome of other human parasites, including Plasmodium spp., Schistosoma spp., and Entamoeba histolytica. The enzyme seems, therefore, to be a potential target for broad spectrum anti-parasitic drugs.

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