Mitochondrial Fatty Acid Synthesis in *Trypanosoma brucei* *

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Whereas other organisms utilize type I or type II synthases to make fatty acids, trypanosomatid parasites such as *Trypanosoma brucei* are unique in their use of a microsomal elongase pathway (ELO) for *de novo* fatty acid synthesis (FAS). Because of the unusual lipid metabolism of the trypanosome, it was important to study a second FAS pathway predicted by the genome to be a type II synthase. We localized this pathway to the mitochondrion, and RNA interference (RNAi) or genomic deletion of acyl carrier protein (ACP) and β-ketoacyl-ACP synthase indicated that this pathway is likely essential for bloodstream and procyclic life cycle stages of the parasite. *In vitro* assays show that the largest major fatty acid product of the pathway is C16, whereas the ELO pathway, utilizing ELOs 1, 2, and 3, synthesizes up to C18. To demonstrate mitochondrial FAS *in vivo*, we radiolabeled fatty acids in cultured procyclic parasites with [14C]pyruvate or [14C]threonine, either of which is catabolized to [14C]acyetyl-CoA in the mitochondrion. Although some of the [14C]acyetyl-CoA may be utilized by the ELO pathway, a striking reduction in radiolabeled fatty acids following ACP RNAi confirmed that it is also consumed by mitochondrial FAS. ACP depletion by RNAi or gene knockout also reduces lipoyc acid levels and drastically decreases protein lipoylation. Thus, octanoate (C8), the precursor for lipoyc acid synthesis, must also be a product of mitochondrial FAS. Trypanosomes employ two FAS systems: the unconventional ELO pathway that synthesizes bulk fatty acids and a mitochondrial pathway that synthesizes specialized fatty acids that are likely utilized intramitochondrially.

Trypanosoma brucei, the protozoan parasite that causes nagana in cattle and African sleeping sickness in humans, is transmitted from the tsetse fly to its mammalian host. Two of its life cycle stages are easily cultured in the laboratory: the procyclic form (PCF), which normally resides in the insect midgut, and the bloodstream form (BSF), which dwells in the mammalian host. These two stages inhabit markedly different environments explaining their distinct metabolism. PCF trypanosomes metabolize both sugars and amino acids, depending on what is available in the vector midgut (1, 2), whereas BSF trypanosomes use blood glucose as their primary energy source (3).

Each parasite cell has a single mitochondrion that differs morphologically and metabolically in the two life cycle stages. The PCF mitochondrion is a highly branched tubular network rich in cristae; its inner membrane has a complete respiratory chain (4). BSFs, on the other hand, have a less developed mitochondrion with fewer branches extending through the cell. This atrophied organelle utilizes respiratory components that do not generate a proton gradient across the inner membrane. Instead, the BSF mitochondrion relies on reverse action of the ATP synthase to maintain the mitochondrial membrane potential (5, 6). BSFs excrete pyruvate as the end product of glycolysis and therefore have a diminished need for mitochondrial metabolism (4). In contrast, PCFs use a mitochondrial pyruvate dehydrogenase to decarboxylate pyruvate and form acetyl-CoA. PCFs then utilize the acetate:succinate CoA transferase cycle to convert acetyl-CoA to acetate, which they secrete as the primary metabolic end product (7, 8).

Until recently, very little was known about fatty acid metabolism in either stage of the trypanosome. In fact, for many years it was thought that BSFs could not make fatty acids at all (9). More recently, however, our laboratory reported robust *in vitro* fatty acid synthesis (FAS) activity using BSF membranes as the source of enzymes (10). In this life cycle stage, the product of this pathway is predominantly myristate (C14), which can be incorporated into the glycosylphosphatidylinositol anchor of the variant surface glycoprotein (11). In contrast, for PCFs, the predominant fatty acid produced *in vitro* is stearate (C18) (10).

A recent objective of our laboratory has been to identify the enzymatic machinery used for trypanosome FAS. In other organisms, there are two types of FAS, which catalyze similar reactions but differ in structure. Type I synthases, found in the cytosol of eukaryotes, have each enzymatic activity on a separate domain of one or two giant polypeptides (12). Type II synthases, in contrast, are found in bacteria and in eukaryotic organelles of bacterial origin and have similar enzymatic activities residing on separate polypeptides (13). In both systems the growing acyl chain, linked to acyl carrier protein (ACP) or an ACP-like domain, is condensed with malonyl-ACP by β-ketoacyl-ACP synthase (KAS). The β-ketoacyl-ACP product is then reduced by β-ketoacyl-ACP reductase (KAR), dehydrated by β-hydroxyacyl-ACP dehydrase, and reduced again by trans-2-enoyl-ACP reductase (ENR). The reducing agent in both reduction steps is NADH or NADPH, and the product after each round of synthesis is a saturated acyl chain extended in length by two carbon atoms.
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The trypanosome genome encodes only a type II system, so we initially assumed that it was responsible for the observed in vitro FAS activity. Instead we found that trypanosome FAS is unconventional and that they make most of their fatty acids using a set of microsomal elongases (14). In other eukaryotes, including mammals, elongases extend pre-existing long chain fatty acids such as palmitate (C16) (15). Remarkably, trypanosomes have adapted this system for de novo synthesis. The chemistry of the elongase reactions is the same as that for type I and II systems except that malonic acid and the growing acyl chains are esterified to coenzyme A (CoA) instead of ACP. The type I and II systems except that malonic acid and the growing acyl chain are esterified to coenzyme A (CoA) instead of ACP. The trypanosome elongase activity in vitro utilizes butyryl-CoA as the primer, malonyl-CoA as the two-carbon donor, and NADPH as the reducing agent (10, 14). There are three elongases (ELO1–3) responsible for the stepwise synthesis of saturated fatty acids. Although they have somewhat overlapping specificities, ELO1 elongates C4 to C10, ELO2 converts C10 to C14, and then ELO3 extends C14 to C18. Additionally, they are regulated throughout the life cycle of the trypanosome. For example, down-regulation of ELO3 in BSFs allows the production of myristate (14).

If the ELO system makes most of the fatty acids needed by trypanosomes, what is the function of the type II synthase? To address this question, we searched the genome to identify candidate genes and localized their gene products to the mitochondrion. We then showed that C8 (the precursor for lipoic acid synthesis) and C16 are products of the pathway. Finally, we genetically modified the trypanosomes and found that the mitochondrial type II pathway is likely essential in both life cycle stages. This finding indicates a greater role for mitochondrial metabolism, especially in BSF trypanosomes.

EXPERIMENTAL PROCEDURES

Trypanosomes—T. brucei brucei PCF trypanosomes, strains 927, 29-13 (16), and derivatives, were maintained at 28 °C and 5% CO2 in SDM-79 medium (17) supplemented with 10% fetal bovine serum (unless otherwise noted) and antibiotics as needed (15 μg/ml G418, 50 μg/ml hygromycin, and 2.5 μg/ml phleomycin). T. brucei BSF trypanosomes, strain 427, the single marker line (18), and derivatives, were maintained at 37 °C and 5% CO2 in HMI-9 medium (19) supplemented with 10% fetal bovine serum and 10% Serum Plus with antibiotics as needed (2.5 μg/ml G418, 5 μg/ml hygromycin, 2.5 μg/ml phleomycin, 5 μg/ml blasticidin, and 0.1 μg/ml puromycin). All trypanosomes, with the exception of PCF 927, were from Dr. George Cross of Rockefeller University. PCF 927 was from Dr. Elisabetta Ullu of Yale University.

Recombinant ACP, Antibody Production, and Immunofluorescence—Full-length ACP (including its mitochondrial targeting signal) tagged at the carboxyl terminus with 6× His (using the pET-22b vector from Novagen) was expressed in Escherichia coli (Novagen Rosetta (DE3) pLysS strain) in 1 liter of Luria Broth containing chloramphenicol (34 μg/ml) and carbenicillin (50 μg/ml). Expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4–5 h at 37 °C. Virtually all of the recombinant ACP was sequestered in inclusion bodies,3 which were purified essentially as described (20). Briefly, they were suspended in a buffer containing 2 M urea and 0.1% Triton X-100, washed several times, and then stored in 4 mM dithiothreitol at −80 °C. For recombinant ACP purification, the inclusion bodies were resuspended in solubilization buffer (6 M guanidine-HCl, 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole, and 0.1 mM phenylmethylsulfonfluoride) and passed through a 1-ml nickel column (Qiagen nickel-nitritriacetic acid-agarose). To favor refolding of the protein, the column was washed with 10 ml of a reverse gradient of 6 M to 1 M urea in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole, and 0.1 mM phenylmethylsulfonyl fluoride. The recombinant ACP was then eluted with two column volumes of 500 mM imidazole, pH 8.0. The purification yielded roughly 10 mg of recombinant ACP from 1 liter of culture, and SDS-PAGE of the product (Fig. 1A) revealed a near homogeneous polypeptide of the predicted size for the full-length protein (17 kDa).

To generate antibodies, recombinant ACP was resolved on a 12% SDS-polyacrylamide gel, excised, and submitted for commercial antibody production in rats (Cocalico Biologicals). For immunofluorescence, the resulting antibody was diluted 1:50 and used to stain trypanosomes following fixation as previously described (21). AlexaFluor 488 goat anti-rat IgG (Molecular Probes) was used as the secondary antibody (diluted 1:250), and the resulting fluorescence was viewed on a Zeiss Axioskop microscope. Mitochondrial lipoamide dehydrogenase was visualized using an antibody provided by Dr. Luise Krauth-Siegel (University of Heidelberg) as previously described (22).

Enzyme Localization Using Myc and GFP Tagging—The full-length sequence of KAS (see Table 1 for accession number) was cloned into the pLEW-TAP vector (23, 24), which expressed the gene product with a carboxyl-terminal TAP tag (includes a Myc epitope) downstream of a procyclin promoter under control of a tetracycline operator. Full-length KAR1, KAR2, mENR1, and mENR2 were each cloned into pLEW111–21T7GFP (25), which expressed the gene product with a carboxyl-terminal GFP tag. After transfection into PCF (29–13) trypanosomes that express T7 RNA polymerase and tetracycline repressor, production of the Myc- or GFP-tagged proteins was induced using tetracycline (5 μg/ml for KAS-Myc and 0.2–2 μg/ml for GFP fusions). To visualize KAS-Myc, cells were harvested 4–6 days after tetracycline induction, fixed as previously described (21), and examined by immunofluorescence using rabbit anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 and AlexaFluor 594 goat anti-rabbit IgG (Molecular Probes, diluted 1:500) as the secondary antibody (21). For KAR and mENR-GFP fusion proteins, ~1 × 107 cells were harvested after 1–2 days of expression, washed with 1 ml of PBS, and imaged live using fluorescence microscopy as described (25). For mitochondrial staining, ~1 × 107 cells were incubated (10 min at 27 °C) with 40 nM MitoTracker Red (Molecular Probes), washed, and resuspended in the original volume of SDM-79 medium, incubated again (20 min at 27 °C),

3 Although sequestered in inclusion bodies, the E. coli phosphopantetheinyl transferase (which adds the prosthetic group to ACP) may not have access to recombinant ACP, thus leaving most of the protein in its apo-form.
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RNAi and Knock-out Constructs—Full-length ACP was cloned into pZJM, a tetracycline-inducible RNAi vector that uses opposing T7 promoters to produce double-stranded RNA (26). Following transfection into PCF (29-13) trypanosomes, the cells were cloned by limiting dilution in the appropriate medium supplemented with 15% fetal bovine serum. For ACP knock-out construction in BSF trypanosomes, ~500 bp each of the 5’ and 3’ untranslated regions from the ACP genomic sequence was inserted into pKo (27) (a gift from Dr. Jay Bangs, University of Wisconsin) containing resistance markers for blasticidin (BSR) or puromycin (PUR). The first ACP allele was replaced with pKOBSR in the BSR single marker line (expresses mitochondria). Attempts to replace the second allele were unsuccessful unless we first introduced an ectopic copy of the gene (ACPec). For ACPec construction, the entire ACP gene (including a stop codon between the gene and the TAP tag) was inserted into the pLEW-TAP vector as above for KAS-Mycc expression. ACPec was transfected into pKOBSR trypanosomes, and expression was maintained by including 1 μg/ml tetracycline in the growth medium. ACPec expression allowed replacement of the second Acp allele with pKOPUR.

Fatty Acid Synthesis in Isolated Mitochondria—PCF trypanosomes (1 × 10^10 cells in log phase growth) were lysed hypotonically in 20 ml of 1 mM Tris-HCl, pH 7.9, 1 mM EDTA, and mitochondria were purified on a 20–35% renografin gradient (28). The mitochondrial fraction was washed and resuspended in a FAS buffer (0.25 M sucrose, 20 mM Tris-HCl, pH 7.9, 10 mM MgCl_2, and protease inhibitors (Complete, 1 tablet/100 ml, Roche Applied Science)). Mitochondrial integrity was assessed (in a similar experiment) using 50 μg/ml DAPI, which stains mitochondrial DNA, and by determining the enrichment of HSP70, a mitochondrial matrix enzyme (detected by Western blot using a polyclonal rabbit antibody (1:4000) generated against Crithidia fasciculata mitochondrial HSP70 (29)).

Isolated mitochondria (150 μg of protein, 1 × 10^9 cell equivalents) in 30 μl of FAS buffer were either immediately assayed for 30 min at 37°C in the presence of 1 mM ATP and 50 μM [2-14C]malonyl-CoA (American Radiolabeled Chemicals, 55 mCi/mmol) or preincubated to assess heat lability of FAS activity. Preincubation was for 10–30 min at 37°C (without ATP and malonyl-CoA). This was followed by an additional 30 min at 37°C (with ATP and malonyl-CoA). Some reactions contained 50 μM butyryl-CoA as a potential primer. Lipids were then extracted in chloroform/methanol/water (10:10:3) and converted to methyl esters for chain-length analysis by fractionation on reversed-phase TLC (10, 14, 30). TLCs were exposed to a phosphorimaging screen and quantitated on a FujiFilm BAS-2500 phosphorimaging device. All values (arbitrary units) were corrected for background and normalized for protein content.

Fatty Acid Synthesis Assays Using Total Lysates—BSF knockouts of either ELO1 or -3 (14) were hypotonically lysed (5 × 10^8 cell equivalents/ml) as previously described (10, 14, 30), except cells were grown in culture (instead of in rats) and lysates were incubated for 20 min on ice before being restored to isotonic conditions. In addition, the membrane-washing steps used previously were omitted; instead, total lysates were used.

Assays were conducted (within 2 h of cell lysis) in 30 μl of 50 mM HEPES-KOH, pH 7.5, 25 mM KCl, 10 mM MgCl_2, with 2 mM NADH, 2 mM NADPH, 1 mM ATP, 100 μM CoA, 50 μM [2-14C]malonyl-CoA (American Radiolabeled Chemicals, 55 mCi/mmol), and protease inhibitors (Complete, 1 tablet/100 ml, Roche Applied Science). Substrates and cofactors (NAD(P)H, ATP, CoA, and malonyl-CoA) were left out during preincubation (as done for isolated mitochondria). Some reactions contained 50 μM butyryl-CoA as a primer. After 30 min at 37°C, lipids were extracted in chloroform/methanol/water (10:10:3) and used for chain-length analysis and quantitated as described earlier in this section.

In Vivo Labeling of ACP RNAi Cell Lines—ACP RNAi was induced with 1 μg/ml tetracycline. For 4 days, cells (induced and uninduced controls) were centrifuged and resuspended at 2 × 10^8 cells/ml in SDM-79 medium with the appropriate drugs. This cell suspension (500 μl) was transferred to a screw-cap tube with either 1.2 μCi of [2-14C]pyruvate (PerkinElmer Life Sciences, 16.4 mCi/mmol) or 3 μCi of U-14C]threonine (Sigma, 155 mCi/mmol) for 4 h at 27°C under 5% CO_2 (with cap loosened). Radiolabeled cells were centrifuged, and medium was saved for scintillation counting. The pellet was then washed in PBS, and fatty acids were extracted and analyzed by normal-phase TLC (10) and for acyl chain-length by reversed-phase TLC (14). Phosphorimaging quantitation was performed as above, but values were normalized using scintillation counts from labeling medium.

Lipoic Acid Bioassays—The E. coli lipoate synthase null mutant, KER176 (provided by Dr. Sean Prigge, Johns Hopkins University), was plated on Luria Broth with kanamycin (50 μg/ml) and a single colony was selected. The cloned cells were cultured at 37°C in medium E (31) supplemented with 0.4% glucose, 1 mg/ml acid hydrolyzed vitamin-free casein (Difco), 7 μg/ml iron sulfate, 2 μg/ml thiamine hydrochloride, and 10 ng/ml LA (complete medium E). Prior to the assay, cells were washed in 0.9% NaCl and resuspended in 10 ml of complete medium E without LA and grown overnight at 37°C. Cells were then harvested and washed twice in 0.9% NaCl before resuspension to an A_660 nm of 0.2 for inoculation.

Trypanosomes (3 × 10^8 cells) either uninduced or induced for ACP RNAi (PCFs) or knock-out (BSFs) for 4 days were harvested and resuspended in 0.3 ml of 6 mM HCl containing 3 mg of bovine serum albumin in a screw-cap tube. A control tube contained no trypanosomes. Samples were saturated with nitrogen gas for 20 min and then heated in an autoclave (120°C for 20 min (32)). The resulting hydrolysates were dried under nitrogen gas and redissolved in 0.3 ml of 200 mM potassium phosphate, pH 7.0.

Hydrolysate (1 × 10^8 cell equivalents) was added to 3 ml of complete medium E (without LA) containing kanamycin and 100 μl of KER176 inoculum. After 18 h at 37°C, cell growth was determined by measurement of A_660 nm. A standard curve, generated by adding LA to the culture instead of trypanosome hydrolysate, was linear up to 3 ng of LA.

Northern Blots and Western Blots—Northern blots were performed as previously described (26), except 2 × 10^7 cell equiv-
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TABLE 1
Candidate type II FAS proteins encoded by the T. brucei genome

| Protein | Abbreviation | Accession no. | Yeast homolog* | Predicted amino acids | % identity (no. of amino acids)* | Localization |
|---------|--------------|---------------|----------------|-----------------------|----------------------------------|-------------|
| β-Ketoacetyl-ACP synthase | KAS | AAX79622 | CEM1 (49) | 500 | 33% (494) | Mito |
| β-Ketoacetyl-ACP reductase | KAR1 | AAX79999 | OAR1 (67) | 241 | 34% (129) | Mito |
| | KAR2* | AAX69287 | | 498 | | Mito |
| | KAR3* | AAX69289 | | 474 | | Mito |
| Enoyl-ACP reductase | mENR1* | XP_827075 | ETR1 (68) | 445 | 21% (201) | Mito |
| | mENR2* | AAX79977 | ETR1 (68) | 335 | 22% (245) | Mito |
| Acyl carrier protein | ACP | AAX69898 | ACP1 (57) | 148 | 50% (100) | Mito |

* Yeast mitochondrial FAS enzymes and reference.
* Percent identity to yeast homolog over indicated number of amino acids.
* Homologous to β-ketoacyl-CoA reductases of the ELO pathway (KAR2, 31% over 71 amino acids; KAR3, 35% over 78 amino acids).
* The “m” designation distinguishes these enzymes from the ENR involved in the elongase pathway (14).

RESULTS

Genomic Evidence for a T. brucei Type II Fatty Acid Synthase—By searching the T. brucei genome, we identified candidate genes encoding nearly all the components of a type II FAS pathway (Table 1). There are single KAS and KAR genes (KAR1) with homology to the yeast mitochondrial β-ketoacyl-ACP synthase, CEM1, and β-ketoacyl-ACP reductase, OAR1. There are two additional KAR genes (KAR2 and KAR3) in tandem on chromosome 8 that may also function in the type II FAS pathway. These two genes are related to others from the short-chain dehydrogenase family, which encompasses many other β-ketoacyl reductases, and their amino acid sequences have strongly predicted mitochondrial targeting signals. Two more genes (mENR1 and mENR2) are 20% identical to their yeast mitochondrial counterparts, ETR1. Even though the homology with ETR1 is weak, mENR1 and mENR2 are more homologous to mammalian and plant mitochondrial ENRs (up to 31% over 282 amino acids). There is a single ACP gene that is most homologous to the chloroplast ACP1 of Arabidopsis thaliana (62% identity over 82 amino acids) but is also similar to yeast mitochondrial ACP1 (Table 1). Due to low sequence conservation among different species, we have not yet identified a trypanosome gene for the remaining enzyme in type II FAS, β-hydroxyacyl-ACP dehydrase. Additional enzymes that attach FAS precursors to ACP have been previously identified or can be found in the genome; a single malonyl-CoA:ACP transacylase is predicted to localize to the mitochondrion (33), and there are seven acyl- and acetyl-ACP transferase-like proteins (three of which have candidate mitochondrial targeting signals).

Localization of Type II Fatty Acid Synthase Enzymes—Our next objective was to identify the intracellular location of the candidate enzymes listed in Table 1. Although Mitoprot (ihg.gsf.de/ihg/mitoprot.html) and Psort (psort.hgc.jp) software predicted that most are mitochondrial, we localized them experimentally using fluorescence microscopy. For ACP, we performed immunofluorescence with an antibody against recombinant ACP (rACP). The third lane is a Western blot (from a separate gel) of PCF lysates (lys 1 × 10^7 cell equivalents) using the polyclonal rabbit antibody produced against rACP. Although rACP is 17 kDa (as predicted by its coding sequence), the size of the ACP detected in lysates is 10 kDa (**, processing of the 17-kDa form may be due to in vivo processing or, despite precautions, proteolysis during sample preparation). This 10-kDa species is the only protein detected in whole cell lysates, and it is lost following ACP RNAi or knockout. B, fluorescence images from left to right, anti-recombinant ACP, anti-lipoamide dehydrogenase (LipDH), and DAPI staining. N, nucleus; K, kinetoplast (this cell had undergone kinetoplast division). C, fluorescence images from left to right, anti-Myc, anti-recombinant ACP, and 4′,6-diamidino-2-phenylindole staining. D, top panels show localization using GFP fusion proteins of KAR1, KAR2, KAR3, mENR1, and mENR2 as indicated; bottom panels show MitoTracker Red fluorescence (MiTr) of the same cell.

FIGURE 1. Localization of type II FAS proteins in PCF trypanosomes. A, Coomassie-stained SDS-PAGE gel showing size markers (M) and 2 μg of purified recombinant ACP (rACP). The third lane is a Western blot (from a separate gel) of PCF lysates (lys 1 × 10^7 cell equivalents) using the polyclonal rabbit antibody produced against rACP. Although rACP is 17 kDa (as predicted by its coding sequence), the size of the ACP detected in lysates is 10 kDa (**, processing of the 17-kDa form may be due to in vivo processing or, despite precautions, proteolysis during sample preparation). This 10-kDa species is the only protein detected in whole cell lysates, and it is lost following ACP RNAi or knockout. B, fluorescence images from left to right, anti-recombinant ACP, anti-lipoamide dehydrogenase (LipDH), and DAPI staining. N, nucleus; K, kinetoplast (this cell had undergone kinetoplast division). C, fluorescence images from left to right, anti-Myc, anti-recombinant ACP, and 4′,6-diamidino-2-phenylindole staining. D, top panels show localization using GFP fusion proteins of KAR1, KAR2, KAR3, mENR1, and mENR2 as indicated; bottom panels show MitoTracker Red fluorescence (MiTr) of the same cell.

alents of total RNA were loaded per lane. For anti-ACP Western blots, 2 × 10^7 cells (unless otherwise noted) were harvested per day and stored at −80 °C until fractionated on a 15% SDS-PAGE gel. The proteins were then transferred to a polyvinylidene difluoride membrane and incubated with rat anti-recombinant ACP (1:500) followed by donkey anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, 1:2000) followed by donkey anti-rabbit horseradish peroxidase antibody (Pierce, 1:5000) and detected by ECL. For anti-lipoate Western blots, ACP RNAi was induced and 1 × 10^7 cells were harvested per day and stored at −80 °C until fractionated on a 12% SDS-PAGE gel. After transfer, the membrane was probed with rabbit anti-lipoate antibody (Calbiochem, 1:2000) followed by donkey anti-rabbit horseradish peroxidase antibody (Pierce, 1:5000) and detected by ECL.
FIGURE 2. Depletion of ACP in PCF and BSF trypanosomes. A, growth curves, typical of results from at least five independent experiments, show the effect of ACP RNAi in PCF (left panel) and down-regulation of the ACP ectopic copy (ACPec) in the BSF ACP knockout (right panel). RNAi and ACPec down-regulation were induced by addition or removal, respectively, of 1 μg/ml tetracycline at day 0. Parasites/ml (Y-axis) was calculated by multiplying cell density and dilution factor. B, autoradiograph of Northern blot of total RNA from uninduced (−RNAi) and induced (+RNAi, 1 day) PCF RNAi cells. ACP mRNA is −0.9 kb and double-stranded RNA (dsRNA) is −0.7 kb. Load control is an ethidium-stained rRNA species. C, Western blot detection of ACP following RNAi in PCF or down-regulation of ACPec expression in the BSF knockout. In these experiments, the 10-kDa species was detected and depleted (see Fig. 1A). The load control is a portion of the Coomassie-stained gel (stained following transfer to membrane).

with an anti-Myc antibody. KAS-Myc colocalized with ACP (Fig. 1C), which resides in the mitochondrion (Fig. 1B). For KAR and ENR, we expressed fusion proteins that had GFP at their carboxyl termini. GFP fusions of KAR1, KAR2, KAR3, mENR1, and mENR2 all colocalize with MitoTracker Red staining in PCFs (Fig. 1D). These data confirm that all of the proteins listed in Table 1 are indeed mitochondrial.

RNAi and Knockout of Acyl Carrier Protein—To investigate whether mitochondrial FAS is essential in trypanosomes, we used RNAi to silence expression of PCF RNAi. These trypanosomes cease growth after 3 days of RNAi (Fig. 2A, left panel). A Northern blot showed a 10-kDa mRNA below detectable levels after 24 h (Fig. 2B) and a Western blot barely detected ACP protein after 3 days (Fig. 2C, upper set). During ACP RNAi, the PCF trypanosomes did not divide yet persisted in the culture. As with RNAi on many other trypanosome genes, induced cells recovered after 7–8 days of RNAi, and their growth rate returned to normal (data not shown). Northern blot analysis on the recovered cells revealed a decrease in the level of double-stranded RNA and reappearance of the ACP mRNA (data not shown).

Although effective, RNAi did not reduce ACP mRNA levels low enough to affect BSF growth (data not shown). Therefore, we prepared a conditional knockout of ACP in which the two endogenous ACP alleles were removed (ΔACP) while the cells were sustained by expression of an ectopic copy of the gene (ACPec) under control of a tetracycline operator. Down-regulation of ACPec expression by removal of tetracycline caused BSF trypanosomes to stop dividing after 3 days (Fig. 2A, right panel) following a nearly complete loss of ACP protein after 2 days (Fig. 2C, lower set). Unlike ACP RNAi in PCFs, elimination of ACPec expression in BSFs resulted in death of nearly all the cells (as judged by lysis and clumping). After several days without tetracycline, however, some apparently normal cells appeared in the culture, presumably because they lost regulation of ACPec.

We added fatty acids to cultures in an attempt to rescue PCF cells after ACP RNAi or BSF cells after ACP knockout. Adding myristate (C14:0), palmitate (C16:0), or stearate (C18:0), either alone or in combination at concentrations ranging from 1 to 100 μM, did not prevent or reverse the growth arrest (data not shown).

Fatty Acid Synthesis in Isolated Mitochondria—After fractionation of PCF cell lysate on a renografin gradient, the mitochondrial fraction contained many intact vesicles with DAPI-staining mitochondrial DNA. Compared with other fractions of the gradient, our target fraction was enriched 5-fold in the mitochondrial matrix enzyme, HSP70, relative to total protein (data not shown). Nevertheless, some mitochondria must have broken during isolation; a rough quantitation of HSP70 distribution throughout the gradient attributed roughly 60% of the protein to the mitochondrial fraction, 25% in the pellet (which includes unbroken cells), with 15% in remaining fractions.

To identify the products of PCF mitochondrial FAS, we radiolabeled fatty acids in isolated mitochondria using a [14C]malonyl-CoA precursor. Like mitochondrial FAS assays described for plants (35), we added 1 mM ATP to these assays. NAD(P)H (2 mM) did not enhance our FAS activity and was therefore not included. The radiolabeled FA products were converted to methyl esters, fractionated by reverse-phase TLC, and detected by phosphorimaging. In a control experiment using PCF total lysates (prepared like BSF total lysates, see “Experimental Procedures”), a butyryl-CoA primer was readily extended to C18 by the microsomal ELO pathway (Fig. 3A, lane 1). As shown in Fig. 3A (lanes 2 and 3), fatty acid products from isolated mitochondria ranged from C10 to C16 (the shorter products most likely being intermediates in the synthesis of C16). These mitochondrial products were synthesized at comparable levels regardless of the presence (lane 2) or absence (lane 3) of a butyryl-CoA primer. Thus it appears that, in this assay system, isolated mitochondria do not use an exogenous primer; instead, they must utilize a primer that is supplied endogenously. An attempt to increase access of exogenous primers to FAS enzymes by disruption of mitochondria did not enhance activity. In fact, FAS activity was drastically reduced following addition of non-ionic detergent (0.3–1% Triton X-100). Loss of activity could be due to enzyme dilution, although we could not recover FAS by adding molecular crowding agents (6% polyethylene glycol 8,000 or 20,000 (36)). Lastly, this butyryl-CoA-independent activity from isolated mitochondria is almost completely lost during a 30-min preincubation at 37 °C, whereas butyryl-CoA-dependent ELO activity was stable (Fig. 3B). This observation, along with the disparity
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A BSF cell line lacking the gene for the first enzyme of the ELO pathway (ΔELO1) exhibited markedly reduced fatty acid synthesis using a butyryl-CoA primer (14). Indeed, when we assayed ΔELO1 lysates under our new conditions (with butyryl-CoA) and analyzed products for their chain-length, there was a drastic loss in production of shorter chain fatty acids when compared with wild-type ELO activity; products ranged from C8 to C18 in wild-type lysates (Fig. 3C) but were predominantly C16 and C18 in ΔELO1 lysates (lane 2). If the butyryl-CoA primer was omitted causing synthesis to rely on endogenous primers, the same amount of product and chain length profile was produced in both wild-type (Fig. 3C, lane 3) and ΔELO1 lysates (lane 4). In agreement with our observations on isolated mitochondria, products from ΔELO1 lysates (both C16 and C18) were reduced ~85% within the first 10 min of preincubation at 37 °C, conditions in which ELO activity is stable (Fig. 3D). These results support our hypothesis that mitochondrial FAS contributes to the activity detected in BSF ΔELO1 lysates.

Nevertheless, we considered the possibility that the C16 and C18 synthesized in the ΔELO1 lysates could be due to ELO3 activity on endogenous fatty acids (ELO3 converts C14 and C16 to C18 (14)). We therefore assayed both ΔELO1 and ΔELO3 lysates for butyryl-CoA-independent activity (Fig. 3E). Compared with ΔELO1 lysates (Fig. 3E, lane 2), production of C18 (but not C16) was diminished ~90% in the ΔELO3 lysates (Fig. 3E, lane 3). Because deletion of ELO3 (and also ELO2; data not shown) had no effect on C16 levels, this fatty acid is likely not a product of mitochondrial FAS. Instead, we considered the possibility that short-chain fatty acids (C16 and C18) could provide additional energy to sustain the parasitic lifestyle in the mammalian host. These results provide a new avenue to probe the metabolic strategies employed by T. brucei to sustain its intracellular lifestyle.

in chain length of the principal fatty acid products, provides strong biochemical evidence that the activity detected in isolated mitochondria is not due to contamination by the microsomal ELO pathway. Despite these results, FAS activity in isolated PCF mitochondria was low. A mitochondrial fraction from 1 × 10^10 PCF cell equivalents (derived from 2 liters of culture) was sufficient for only 10 FAS assays (using a 14-day phosphorimaging exposure). Enzyme lability during the 8-h isolation, mitochondrial breakage, and reduced recovery of organelles could have contributed to the low activity of our preparations.

Fatty Acid Synthesis in Lysates Prepared from ELO Knockouts—As an alternative assay of mitochondrial FAS in vitro, we turned to lysates in which the ELO system had been deleted by gene knockout. Lysate preparation for these experiments resembled methods previously used to assay ELO activity (10, 14, 30), except washed membranes were replaced with total lysates (which were 40% more active than washed membranes (data not shown)). For these assays, we often left out exogenous primers, normally required for ELO activity, and we included NAD(P)H, ATP, and CoA, which individually enhanced mitochondrial FAS by 2-, 4-, and 2-fold, respectively (data not shown). As mentioned previously, NAD(P)H supplementation did not enhance FAS activity in isolated mitochondria implying that these nucleotides may be acting on some unknown cytosolic reaction (present in total cell lysates), which indirectly increases mitochondrial FAS activity.

Mitochondrial FAS activity in total lysates (from BSFs) was several hundred-fold higher than in isolated mitochondria (from PCFs); lysates from 1 × 10^8 BSF cell equivalents (derived from 2 liters of culture; note: BSFs can only be grown to 1/10th the density of PCFs) could support 50 FAS assays (using only a 1-day phosphorimaging exposure). Furthermore, upon analysis of supernatants (by FAS assays and Western blots) following centrifugation of total lysates (14,000 rpm, 10 min, 4 °C), we found no FAS activity and <10% of the HSP70 protein, implying minimal mitochondrial breakage. The disruption of membranes using sonication or freeze-thaw did not liberate FAS components into the supernatant following centrifugation (as above).
product of the ELO pathway. Thus experiments using lysates defective in the ELO pathway agree with those using isolated mitochondria: the largest major product of mitochondrial FAS is C16.

In Vivo Precursors of Mitochondrial Fatty Acid Synthesis—To examine the role of mitochondrial FAS in vivo, we labeled cultured trypanosomes with fatty acid precursors. Radiolabeled acetate, the conventional FAS precursor (after conversion to malonyl-CoA), is not taken up by *T. brucei* (10). Instead we used [14C]threonine (37–40) or [14C]pyruvate to label fatty acids. After uptake, these two compounds are transported to the mitochondrion where they are converted to [14C]acetyl-CoA (P, lanes 1 and 2) or [14C]threonine (T, lanes 3 and 4) with or without ACP RNAi induction (+ or −RNAi). There were 1 × 10⁷ cell equivalents per lane. A, chain lengths of fatty acid products from the experiment in A (lanes 1 and 3). M, marker consisting of ELO FAS products. HPL, unknown hydrophilic species.

We first measured the effect of ACP RNAi on FAS using [14C]pyruvate as a precursor. Addition of [14C]pyruvate to cultured wild-type PCF trypanosomes resulted in efficient incorporation of radiolabel into free fatty acids, neutral lipids, and phospholipids (Fig. 4A, lane 1). Chain length analysis of the products revealed fatty acids ranging from C12 to C18 (Fig. 4B, lane 2). There was also incorporation into an unknown hydrophilic species that does not comigrate with C8 or lipoic acid standards (data not shown) but could be an intermediate in lipoic acid synthesis. A similar labeling with [14C]pyruvate after 4 days of ACP RNAi (Fig. 4A, lane 2) resulted in a 85% decrease in free fatty acids, neutral lipids, and phospholipids as quantitated by phosphorimaging.

We also studied the effect of ACP RNAi on the incorporation of radioactivity from [14C]threonine into fatty acids. The products (Fig. 4B, lane 3) were similar to those formed using [14C]pyruvate as a precursor. RNAi reduced [14C]threonine labeling of free fatty acids, neutral lipids, and phospholipids by ~45% (Fig. 4A, compare lanes 3 and 4).

**TABLE 2**

| Life cycle stage | Lipoic acid levela | −ACP |
|------------------|--------------------|------|
| PCF | 3.2 ± 0.01 | 1.4 ± 0.05 |
| BSF | 0.6 ± 0.08 | 0.2 ± 0.04 |

| α-LA load | −ACP |
|----------|------|
| day: 0 | 40 kDa |
| 1 | 2 | 3 | 4 | 5 |

**FIGURE 5. Effects of ACP loss on lipoic acid metabolism.** Effect of ACP RNAi on protein lipoylation as determined by Western blot in PCF trypanosomes using anti-LA antibody. ACP RNAi was induced at day 0. Except for the 40-kDa protein shown in the upper panel, no other proteins were detected by anti-LA on this blot. The load control is a portion of the Coomassie-stained gel (stained following transfer to membrane). A slight increase of the lipoylated protein on day 5 is likely due to recovery from RNAi (see “Results”).
same electrophoretic mobility was also drastically reduced in the BSF knockout (data not shown).

DISCUSSION

We have investigated a type II FAS pathway in the mitochondrion of T. brucei. Similar to other mitochondrial type II synthases (47–49), trypanosomes utilize a single KAS to catalyze the condensation step of the FAS cycle. Multiple KARs and ENRs (Table 1), on the other hand, are not typically found in mitochondria, raising the possibility that the products of some of these genes do not take part in the synthesis of fatty acids. Their multiplicity, however, is conserved in the related parasites Trypanosoma cruzi and Leishmania major. Additionally, multiple ENRs that prefer distinct chain lengths of fatty acids and use either NADH or NADPH have been described in E. coli (50), Carthamus tinctorius (safflower seeds) (51), and Euglena gracilis (52). Candida tropicalis, on the other hand, has two mitochondrial ENRs with identical chain length specificities. These enzymes, however, are expressed during different conditions depending on the presence of fermentable or non-fermentable substrates (53).

In addition to verifying that all the proteins listed in Table 1 are mitochondrial (Fig. 1), we also demonstrated by in vivo metabolic labeling that ACP has a direct role in mitochondrial FAS. Nonetheless, our genetic analyses proving that mitochondrial ACP is required for growth of both PCFs and BSFs (Fig. 2A) does not confirm that the entire FAS pathway is essential. There is a possibility that ACP is an essential subunit of respiratory complex I as it is in some fungi, flies, and mammals (54–56) and would therefore be required because of its role in respiration. We wanted to test another component of the mitochondrial FA synthase to assess whether the pathway is indeed indispensable in trypanosomes. KAS was an obvious choice because, as mentioned previously, there is only one copy of this gene. Unfortunately, RNAi on KAS was not effective so we attempted to knock out the gene in BSF. We could not replace both genomic alleles, however, implying that KAS is at least required in this life cycle stage. We tried to introduce an ectopic copy of the gene before replacement of the second allele (as we did with ACP), but we could not obtain a cell line with stable regulation of the ectopic copy. Based on these results, we can only say that mitochondrial FAS is likely essential in both PCFs and BSFs.

To identify the products of mitochondrial FAS, we used in vitro assays with either isolated PCF mitochondria or lysates from BSF ELO knockouts. Both of these conditions showed that the longest predominant fatty acid synthesized by mitochondria is C16. There has been some speculation on the use of mitochondrially synthesized fatty acids in lysophospholipid repair (57) or synthesis of cardiolipin (58). Indeed, we can detect phospholipid-related species whose synthesis may be dependent on mitochondrial FAS (threonine-labeled phospholipids are reduced following induction of ACP RNAi (Fig. 4A, lane 4) and data not shown). Our conclusion that the fatty acids synthesized in the two in vitro assays are produced by the mitochondrial pathway and not by contaminating microsomal ELO activity is based on three observations. First, butyryl-CoA, a robust primer for the ELO pathway, does not increase FAS activity from isolated mitochondria beyond that produced with no additional primer (Fig. 3A, lanes 2 and 3). Second, the major product of mitochondrial FAS (C16) is different from that of the ELO pathway (C18) (Fig. 3, A and E). Third, activity from either isolated mitochondria or lysates from ΔELO1 or ΔELO3 cell lines (whose products are predominantly mitochondrial) is inactivated during preincubation (30 min at 37 °C), whereas wild-type ELO activity is not (Fig. 3, B and D, and data not shown). This inactivation could be due to heat lability of enzymes of the mitochondrial pathway or of enzymes involved in the production, activation, or transport of FAS precursors.

During in vitro FAS assays, radioactivity from exogenous [14C]malonyl-CoA is efficiently incorporated into mitochondrial fatty acids. The mechanism by which this substrate is made in vivo, however, is not yet known. Typically, acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC), producing malonyl-CoA. In trypanosomes, however, there appears to be only a single ACC gene whose product may be cytosolic. Plant mitochondria import malonate (synthesized as malonyl-CoA and then hydrolyzed to free malonate) from the cytoplasm. Then they convert it back to malonyl-CoA using a mitochondrial malonyl-CoA synthetase. Finally, a malonyl-CoA:ACP transacylase transfers the malonate to ACP (43). Trypanosomes may have similar machinery; they encode a candidate mitochondrial malonyl-CoA:ACP transacylase (33) and several proteins homologous to plant malonyl-CoA synthetase. Arguing against this possibility, we cannot label fatty acids with [14C]malonic acid in isolated mitochondria or total lysates. [14C]Pyruvate (which radiolabels fatty acids in vivo (Fig. 4)) also does not label fatty acids in our in vitro assays indicating that hypotonic lysis, used to prepare both isolated mitochondria and total lysates, may damage machinery required for import of FAS precursors. We are therefore pursuing alternative approaches to investigate whether trypanosome mitochondrial FAS uses the same strategy as plants for intramitochondrial malonyl-CoA production.

Another key product of mitochondrial FAS is the C8 precursor of LA, a cofactor of mitochondrial enzymes such as pyruvate dehydrogenase. There is an interconnection between these two mitochondrial pathways; LA synthesis relies on FAS for C8, and FAS relies on pyruvate dehydrogenase, a lipoate-requiring enzyme, for acetyl-CoA. We directly observed this interconnection during metabolic labeling of fatty acids with [14C]pyruvate. During ACP RNAi, incorporation of a [14C]pyruvate into fatty acids was inhibited by ~90%. Such a drastic effect was likely due not only to inhibition of mitochondrial FAS, but also a block in LA synthesis. In the absence of LA, pyruvate dehydrogenase is inactivated, which then prevents conversion of the [14C]pyruvate to [14C]acetyl-CoA. The reduced availability of acetyl-CoA probably affects not only mitochondrial FAS but also microsomal ELO activity. Additionally, a reduction in this metabolite could result in inhibition of other important processes in the mitochondrion, including the acetate:succinate CoA transferase cycle and, subsequently, ATP production via substrate level phosphorylation (7, 59).

Production of [14C]acetyl-CoA from [14C]threonine does not involve a lipoate-requiring enzyme. Thus [14C]threonine-derived [14C]acetyl-CoA is available for use by the ELO pathway (after conversion to malonyl-CoA) even if mitochondrial FAS is
inactivated. Because there are multiple sources of acetyl-CoA and the normal concentration of this metabolite may differ between the mitochondrion and the cytoplasm, it was not possible to determine the relative contribution to total FAS by the ELO and mitochondrial pathways during these experiments.

In an attempt to estimate the contribution of mitochondrial FAS to total fatty acids synthesized in the trypanosome, we used phosphorimaging to measure mitochondrial products (C16 from ELO3 knock-out lysates, Fig. 3E, lane 3) and compare them to total fatty acid products (C8–C18 made by wild-type lysates, Fig. 3E, lane 1). According to this calculation, mitochondrial FAS contributes ~10% to total trypanosome FAS in vitro, with the remainder due to the ELO pathway. The reliability of this calculation is limited by the possibility that these assays may not have been conducted under optimal conditions and that mitochondrial FAS activity is labile. Nevertheless, it does imply that mitochondrial FAS, contrary to other organisms (60), makes a significant contribution to total FAS activity.

During our investigations, we routinely found that lysates of cultured BSF trypanosomes efficiently produced C18 via the ELO pathway (e.g. Fig. 3, C and E, lane 1) rather than stopping at myristate, as reported previously (10). We suggest that the reason for this difference relates to how the lysates were prepared and to the regulation of the ELO pathway (14). In previous studies, trypanosomes had been isolated from the blood of rodents. Under these conditions, ELO3 is apparently down-regulated, because palmitate and stearate (the products of ELO3) can be readily salvaged from the blood. With ELO3 down-regulated, ELOs 1 and 2 produce the myristate that is needed for glycosylphosphatidylinositol anchors (14). In this report, the cell lysates were prepared from BSF trypanosomes cultured in medium containing 10% fetal bovine serum and 10% Serum Plus. Because this medium contains only a fraction of the fatty acids present in whole blood, ELO3 is up-regulated in cells grown in this medium. This explanation is consistent with our previous finding that trypanosomes cultured in medium containing 5% serum efficiently elongate exogenous [3H]myristate to palmitate and stearate, whereas those grown in blood do not (61). In support of this hypothesis, we found that the addition of 70 µM stearate to the culture medium 2 days prior to lysis preparation reduced the amount of newly synthesized stearate in the in vitro assay by ~40% (data not shown).

Herein we report the identification of a mitochondrial FAS pathway in T. brucei. Despite many metabolic differences between the two major trypanosome life cycle stages, our studies revealed several similarities between PFC and BSF mitochondrial FAS; activity relies on an endogenous primer of unknown structure, the chief FA products are C8 and C16, and the pathway appears essential. PFC and BSF metabolism diverges once again in their requirement for LA. Whereas the role of LA in the active mitochondrial metabolism of PFCs is obvious, its function in BSFs is less straightforward. This life cycle stage clearly requires less LA (Table 2) (39, 40) and may therefore survive with reduced mitochondrial FAS (as compared with PFC). In addition, acetyl-CoA (for malonyl-CoA synthesis) in the two life cycle stages may originate from distinct sources. Given the secretion of pyruvate by BSFs, other sources of acetyl-CoA such as threonine and leucine must be readily available. A greater understanding of this metabolic crossroad in BSFs may facilitate the discovery of drugs to treat African sleeping sickness. Recently, research on the structures of type II enzymes (62) has renewed interest in designing more selective drugs against previously validated FAS targets (63, 64). Trypanosomes, however, may turn out to be more susceptible to new classes of natural product-based inhibitors of type II FAS (65, 66).

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