The Initiating Steps of a Type II Fatty Acid Synthase in *Plasmodium falciparum* are Catalyzed by pfACP, pfMCAT, and pfKASIII[†]

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ABSTRACT: Malaria, a disease caused by protozoan parasites of the genus *Plasmodium*, is one of the most dangerous infectious diseases, claiming millions of lives and infecting hundreds of millions of people annually. The pressing need for new antimalarials has been answered by the discovery of new drug targets from the malaria genome project. One of the early findings was the discovery of two genes encoding Type II fatty acid biosynthesis proteins: ACP (acyl carrier protein) and KASIII (β-ketoacyl-ACP synthase III). The initiating steps of a Type II system require a third protein: malonyl-coenzyme A:ACP transacylase (MCAT). Here we report the identification of a single gene from *P. falciparum* encoding pfMCAT and the functional characterization of this enzyme. Pure recombinant pfMCAT catalyzes malonyl transfer from malonyl-coenzyme A (malonyl-CoA) to pfACP. In contrast, $pfACP_{trans}$, a construct of pfACP containing an amino-terminal apicoplast transit peptide, was not a substrate for pfMCAT. The product of the pfMCAT reaction, malonyl-pfACP, is a substrate for pfKASIII, which catalyzes the decarboxylative condensation of malonyl-pfACP and various acyl-CoAs. Consistent with a role in de novo fatty acid biosynthesis, pfKASIII exhibited typical KAS (β-ketoacyl ACP synthase) activity using acetyl-CoA as substrate (k_{cat} 230 min^{-1}, K_M 17.9 $\pm$ 3.4 μM). The pfKASIII can also catalyze the condensation of malonyl-pfACP and butyryl-CoA (k_{cat} 200 min^{-1}, K_M 35.7 $\pm$ 4.4 μM) with similar efficiency, whereas isobutyryl-CoA is a poor substrate and displayed 13-fold less activity than that observed for acetyl-CoA. The pfKASIII has little preference for malonyl-pfACP (k_{cat}/K_M 64.9 $min^{-1}\mu M^{-1}$) over *E. coli* malonyl-ACP (k_{cat}/K_M 44.8 $min^{-1}\mu M^{-1}$). The pfKASIII also catalyzes the acyl-CoA:ACP transacylase (ACAT) reaction typically exhibited by KASIII enzymes, but does so almost 700-fold slower than the KAS reaction. Thiolactomycin did not inhbit pfKASIII (IC_{50} > 330 μM), but three structurally similar substituted 1,2-dithiole-3-one compounds did inhibit pfKASIII with IC_{50} values between 0.53 μM and 10.4 μM. These compounds also inhibited the growth of *P. falciparum* in culture.

Malaria remains one of the most devastating diseases, causing as many as 2.7 million deaths annually with an estimated 400−900 million new cases each year (*1*). Parasite resistance to almost all standard treatment drugs, including chloroquine, atabrine, mefloquine, and pyrimethamine-sulfadoxine, has resulted in a resurgence of malaria and a pressing need for new antimalarials (*2*). Malaria parasites contain an essential organelle called the apicoplast that is thought to have arisen through endosymbiosis of an alga which had previously incorporated a cyanobacterium (*3*, *4*). Due to its prokaryotic origin, the apicoplast contains a range of metabolic pathways that differ radically from those of the host and offer numerous targets for drug therapy using antimicrobial compounds. Recently, several lines of evidence indicate that a dissociated Type II fatty acid synthase (FAS)[1] exists in the apicoplast (*5*−*7*). The discovery of a Type II FAS in *Plasmodium falciparum* is receiving considerable attention as a pathway that can be targeted for chemotherapeutic intervention (*8*−*12*).

In the dissociated Type II FAS, common in plants and bacteria, each reaction is catalyzed by a discrete enzyme (*13*). The Type I synthases found in metazoans are large multifunctional enzymes that contain all of the enzymatic activities on one or two polypeptides (*14*). The initiation of Type II fatty acid biosynthesis requires the acyl carrier protein (ACP) and two enzymes: malonyl-coenzyme A:ACP transacylase

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[1] Abbreviations: FAS, fatty acid synthase; CoA, coenzyme A; DMSO, dimethyl sulfoxide; TLM, thiolactomycin; ec, *Escherichia coli*; pf, *Plasmodium falciparum*; ACP, acyl carrier protein; MCAT, malonyl-coenzyme A:ACP transacylase; KASIII, β-ketoacyl-ACP synthase III; KAR, β-ketoacyl-ACP reductase; HAD, β-hydroxyacyl-ACP dehydratase; ENR, enoyl-ACP reductase; TCA, trichloroacetic acid. A subscript on $pfACP_{trans}$ is used to describe pfACP that contains an amino-terminal apicoplast transit peptide.

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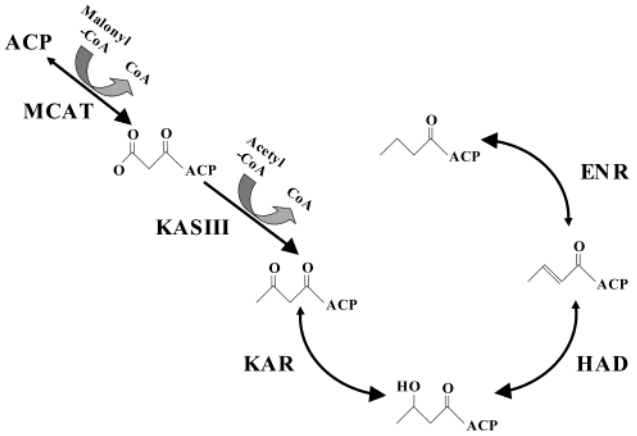

FIGURE 1: Roles of individual enzymes in a Type II fatty acid synthase. The reactions required for initiation and one round of elongation are shown. Full enzyme names are provided in the text.

(MCAT; also called FabD) and β-ketoacyl-ACP synthase III (KASIII; also called FabH) (Figure 1). MCAT produces malonyl-ACP from malonyl-CoA and ACP (*15*−*18*). KASIII catalyzes the condensation of malonyl-ACP and acyl-CoA (typically acetyl-CoA) forming a 3-ketoacyl-ACP product (Figure 1) (*19*−*21*). This product is then reduced to acyl-ACP by the sequential action of β-ketoacyl-ACP reductase (KAR; also called FabG), β-hydroxyacyl-ACP dehydratase (HAD; also called FabA or FabZ), and enoyl-ACP reductase (ENR; also called FabI).

In malaria, the FAS enzymes contain leader peptides that are required to target these nuclear-encoded proteins to the apicoplast organelle. The leader peptides are bipartite, consisting of a signal peptide for entry into the secretory pathway and a transit peptide for subsequent import into the apicoplast. Studies with chimeric mutants show that the ACP and KASIII bipartite leader peptides are necessary and sufficient to target other proteins to the apicoplast (*5*, *6*, *22*). Two mature apicoplast proteins have been purified from cultured parasites and were found to lack the leader peptides, indicating that these amino acids are removed upon import into the apicoplast (*10*, *23*). We have expressed and purified the three *P. falciparum* proteins required to initiate fatty acid biosynthesis (pfACP, pfMCAT and pfKASIII) without their leader peptides. Pure recombinant pfMCAT and pfKASIII catalyze the formation of 3-ketoacyl-pfACP using pfACP, acyl-CoA and malonyl-CoA as substrates, demonstrating for the first time that these processed proteins function as components of a typical Type II FAS. In addition, we have identified several 1,2-dithiole-3-ones which inhibit pfKASIII as much as 2 orders of magnitude more effectively than thiolactomycin.

MATERIALS AND METHODS

Materials. The following reagents were used: *Escherichia coli* acyl carrier protein (ACP), malonyl-CoA, Coenzyme A, S-acetyl-CoA synthetase, ATP, 2-mercaptoethanol, and protein molecular weight standards (Sigma Chemical Company); [1-^{14}C]acetyl-CoA (specific activity 53 mCi/mmol) and [1-^{14}C]butyryl-CoA (specific activity 53 mCi/mmol) (Moravek Biochemicals); [1-^{14}C]malonyl-CoA (specific activity 56 mCi/mmol) (Amersham Biosciences); and [1-^{14}C]-isobutyric acid (specific activity 56 mCi/mmol) (ICN Radiochemicals). Thiolactomycin was a gift kindly provided by Pfizer, and compounds HR12, HR19, and HR45 were obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. All other chemicals were reagent-grade or better and were obtained from VWR Scientific or Fisher Scientific.

Expression and Purification of pfACP. The coding sequence of *P. falciparum* ACP was amplified from a plasmid containing the pfACP described previously (*24*). The primers for pfACP, (F) 5′-GGTGGTGAATTCAGCTCTTTAAA-AAGTACTTTTGATG-3′ and (R) 5′-GGTGGTGTCGACT-TATTGCTTATTATTTTTTTCTATATAATC-3′, were used to amplify nucleotides encoding amino acids 57−137 (amino acids 1−56 contain the putative signal/transit peptide) and introduce a proximal EcoRI and distal SalI site (underlined) in the PCR product. The resulting amplicon was digested with EcoRI and SalI, gel purified, and ligated into the pMALc2x vector (New England Biolabs). The resulting pSTP1 was sequence verified and transformed into BL21 Star(DE3) cells (Invitrogen). These cells were cotransformed with the pRIL plasmid isolated from BL21-CodonPlus(DE3) cells (Stratagene) and used for pfACP expression. Cells were grown in LB medium at 37 °C to an optical density at 600 nm of 0.8 and then induced with the addition of IPTG to a final concentration of 0.4 mM. The culture was maintained in shaker flasks at 20 °C for 12 h and then harvested by centrifugation at 6000*g* for 15 min.

Cell lysis buffer (20 mM Na/K phosphate pH 5.0, 1 mg/mL lysozyme (Sigma), 2.5 μg/mL DNAse I (Sigma), 50 mM NaCl) was added to the cell pellets (20 mL per liter of original culture) and gently vortexed. Resuspended cells were incubated on ice for 10 min followed by 30 s of sonication. Cell lysate was cleared by centrifugation (27 000*g*, 4 °C, 20 min) and applied to a 10 mL amylose column (New England Biolabs) equilibrated in 20 mM Na/K phosphate pH 5.0. The pfACP fusion protein (pfACP fused with the maltose binding protein) bound poorly to the column and was collected while washing the column with 20 mM Na/K phosphate, pH 5.0. Wash fractions were applied to a 5 mL HiTrap Q FF anion exchange column (Amersham Biosciences), equilibrated in 20 mM Na/K phosphate, pH 5.0, and eluted in a linear gradient to 500 mM NaCl. Pure pfACP fusion protein was then digested with factor Xa (New England Biolabs) at a stoichiometry of 1:500 with 1 mM $CaCl_2$ and incubated at 25 °C for 24 h. The reaction mixture was applied to a 10 mL amylose column (New England Biolabs) equilibrated in 20 mM Na/K phosphate, pH 5.0. Cleaved pfACP was collected in the flow through and pooled in a 20 mL 30 000 molecular weight cutoff concentrator (Vivascience). The flow through from the concentrator was then concentrated in a 5 mL 5000 molecular weight cutoff concentrator (Vivascience) to a final concentration of 0.5 mM and flash frozen for further analysis.

A second construct of pfACP encoding amino acids 17−137 ($pfACP_{trans}$) was generated using an alternate forward primer, 5′-GGTGGTGAATTCAAAAATACACAAAAA-GATGGAG-3′. The resulting amplicon was digested with EcoRI and SalI, gel purified, and ligated into a modified version of the pMALc2x vector (pMALcHT) in which the linker region was altered to contain nucleotides encoding a TEV (Tobacco Etch Virus) protease cleavage site, followed

by a six histidine tag. The resulting ligation product, pSTP2 was sequence verified and transformed into BL21 Star(DE3) cells (Invitrogen). These cells were cotransformed with the pRIL plasmid from BL21-CodonPlus(DE3) cells (Stratagene) and a plasmid encoding the TEV protease (*25*). Cells were grown and harvested as described above.

Cell lysate was prepared under the conditions described above and applied to a 5 mL HiTrap Chelating HP column (Amersham Biosciences) equilibrated in 20 mM Na/K phosphate, pH 7.5, 200 mM NaCl. The column was washed with 10 column volumes of 10 mM imidazole, pH 7.5, 200 mM NaCl and eluted with a linear gradient to 500 mM imidazole, pH 7.5. Fractions containing $pfACP_{trans}$ were desalted with a HiPrep 26/10 desalting column (Amersham Biosciences) and loaded on a 5 mL HiTrap SP Fast Flow column (Amersham Biosciences) equilibrated in 20 mM Na/K phosphate, pH 7.5. The column was washed with 10 column volumes of 20 mM Na/K phosphate, pH 7.5, and eluted with a linear gradient to 500 mM NaCl. Fractions containing pure $pfACP_{trans}$ were pooled in a 20 mL 50 000 molecular weight cutoff concentrator (Vivascience). The flow through from the concentrator was then concentrated in a 5 mL 10 000 molecular weight cutoff concentrator (Vivascience) to a final concentration of 0.5 mM and flash frozen for further analysis.

Liquid Chromatography Mass Spectrometry (LC/MS). The electrospray ionization mass spectra were acquired on an Agilent 1100 Series LC/MS quadrupole instrument. Protein samples were electrosprayed from 50% methanolic solution acidified with acetic acid (pH 3.0). The concentrations of the analyte electrospray solutions were in the range of 50−100 μM. Protein solutions were introduced into the ion source via a 300SB-C3 5 μm column (Zorbax) with dimensions 2.1 mm × 150 mm. Samples were loaded on the column in 5% acetic acid and eluted with a linear gradient to 100% acetonitrile. Electrospray ionization measurements were made in the positive mode, and the reconstructed molecular mass profiles were obtained by using a deconvolution algorithm supplied with the instrument data system.

Expression and Purification of pfMCAT. Full-length and truncated coding sequences of *P. falciparum* MCAT were amplified from cDNA generated from asynchronous parasite RNA (3D7 strain). The PCR primer design was based on a putative MCAT gene identified from the Plasmodium genome database PlasmodDB (*26*). The primers for full length pfMCAT ($pfMCAT_{full\ length}$), (F) 5′-GGTGGTGAATTCATGTTTTTATTTAATATGCAAGTAATATTC-3′ and (R) 5′-GGTGGTGTCGACTCATATATATGGAATTGTCCTTTTTG-3′, were used to amplify nucleotides encoding amino acids 1−408 and introduce a proximal EcoRI and distal SalI site (underlined) in the PCR product. An alternate forward primer, 5′-GGTGGTGAATTCAGTTCAGAATATACATTTTTTTTCC-3′, was used to amplify a truncated pfMCAT construct, including the nucleotides encoding amino acids 104−408 (amino acids 1−103 contain the putative signal/transit peptide). PCR products of $pfMCAT_{full\ length}$ and truncated pfMCAT were digested with EcoRI and SalI, gel purified, and ligated into the pMALc2x vector (New England Biolabs) to give pSTP3 and pSTP4, respectively. Both ligation products were sequenced, but expression experiments were conducted using only pSTP4 (pSTP3 was generated to verify the sequence of full length $pfMCAT_{full\ length}$). BL21 Star(DE3)/pSTP4 Cells were generated and transformed with the pRIL plasmid from BL21-CodonPlus(DE3) cells (Stratagene). Cells were grown and harvested as described above.

Cell pellets were resuspended in BugBuster (Novagen) (20 mL per liter of original culture) and the cells were lysed with 30s of sonication. Cell lysate was cleared by centrifugation (27 000*g*, 4 °C, 20 min) and applied to a 10 mL amylose column (New England Biolabs) for purification. The column was washed with 5 column volumes of 20 mM Tris, pH 7.5, 200 mM NaCl and eluted in the same buffer with 100 mM maltose. The pure pfMCAT fusion protein was then desalted with a HiPrep 26/10 Desalting column (Amersham Biosciences) equilibrated in 20 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol. Purified pfMCAT protein was flash frozen in aliquots for further analysis.

pfMCAT Enzyme Activity Assays. Recombinant pfMCAT was assayed for malonyl-coenzyme A:ACP transacylase (MCAT) activity using pfACP and radiolabeled substrate [1-^{14}C]malonyl-CoA in a standard TCA precipitation assay (*27*). The reaction mixture contained 100 mM Na phosphate, pH 7.2, 2 mM 2-mercaptoethanol, 146 μM malonyl-CoA, 23 μM pfACP/$pfACP_{trans}$ and 59 ng pfMCAT in a final volume of 20 μL and was incubated at 37 °C for 30 min.

Expression and Purification of pfKASIII. The coding sequence of *P. falciparum* KASIII was amplified from a plasmid containing this gene, described previously (*24*). The primers for pfKASIII, (F) 5′-GGTGGTGAATTCATGTCCGGAGGTAAAATAATAGGAC-3′ and (R) 5′-GGTGGTGTCGACTCAATATTTAAGTATAACGCATCCATATG-3′, were used to amplify nucleotides encoding amino acids 50−371 (amino acids 1−49 contain the putative signal/transit peptide) and introduce a proximal EcoRI and distal SalI site (underlined) in the PCR product. The resulting amplicon was digested with EcoRI and SalI, gel purified, and ligated into a modified version of the pMALc2x vector (pMALcHT) in which the linker region was altered to contain nucleotides encoding a TEV (Tobacco Etch Virus) protease cut site followed by a six histidine tag. The resulting ligation product, pSTP5, was sequence verified and transformed into BL21 Star(DE3) cells (Invitrogen). These cells were cotransformed with the pRIL plasmid from BL21-CodonPlus(DE3) cells (Stratagene) and a plasmid encoding the TEV protease (*25*). Cells were grown and harvested as described above.

Cell lysis buffer (20 mM Na/K phosphate, pH 7.5, 1 mg/mL lysozyme (Sigma), 2.5 μg/mL DNAse I (Sigma), 200 mM NaCl) was added to the cell pellets (20 mL per L of original culture) and gently vortexed. Resuspended cells were incubated on ice for 10 min, followed by 30 s of sonication. Cell lysate was cleared by centrifugation (27 000*g*, 4 °C, 20 min) and applied to a 5 mL HiTrap Chelating HP column (Amersham Biosciences) equilibrated in 20 mM Na/K phosphate, pH 7.5, 200 mM NaCl. The column was washed with 10 column volumes of 10 mM imidazole, pH 7.5, 200 mM NaCl and eluted with a linear gradient to 500 mM imidazole pH 7.5. Fractions containing the amino-terminal histidine-tagged pfKASIII were desalted with a HiPrep 26/10 desalting column (Amersham Biosciences) and loaded on a 5 mL HiTrap SP Fast Flow column (Amersham Biosciences) equilibrated in 20 mM Na/K phosphate, pH 7.5. The column was washed with 10 column volumes of 20 mM Na/K phosphate, pH 7.5, and eluted with a linear gradient to 500 mM NaCl. Fractions containing pure pfKASIII eluted

with about 150 mM NaCl and were pooled and flash frozen in aliquots for further analysis.

pfKASIII Enzyme Activity Assays. Recombinant pfKASIII was assayed for β-ketoacyl-ACP synthase (KAS) activity using malonyl-pfACP and radiolabeled substrate [1-^{14}C]-acetyl-CoA using a standard TCA precipitation assay (*27*). The malonyl-pfACP for these assays was first generated from malonyl-CoA and pfACP using pfMCAT. The reaction mixture contained 100 mM Na phosphate, pH 7.2, 1 mM 2-mercaptoethanol, 970 μM malonyl-CoA, 153 μM pfACP, and 0.59 μg pfMCAT in a final volume of 30 μL and was incubated at 37 °C for 30 min. The mixture was then aliquoted into pfKASIII assay tubes containing the remaining KAS reaction components except pfKASIII. A standard 20 μL KAS reaction contained the following components: 100 mM Na phosphate, pH 6.6, 2 mM 2-mercaptoethanol, 25 μM [1-^{14}C]acetyl-CoA, 15.4 μM pfACP, 39 ng pfMCAT, 12 ng pfKASIII. The reaction was initiated by addition of pfKASIII and incubated at 30 °C for 10 min. The reaction was terminated and analyzed as described above. The pH dependence of pfKASIII was measured using the assay conditions described above with the pH ranging from 4.2 to 8.0.

Steady-State Kinetics of pfKASIII. Steady-state kinetic parameters for acetyl-CoA, and butyryl-CoA were obtained by determination of pfKASIII activity in the presence of various concentrations of radiolabeled acetyl-CoA (1.88−188.6 μM) or butyryl-CoA (2.38−377.2 μM) in the presence of 11.1 μM malonyl-pfACP. Similarly, an apparent K_M for malonyl-pfACP was obtained by determining pfKASIII activity in the presence of 24.7 μM acetyl-CoA and varying malonyl-pfACP concentrations (0.14−27.7 μM, all malonyl pfACP concentations were based on a 36% conversion rate from pfACP in the preincubation with pfMCAT). Likewise, the apparent K_M for malonyl-ecACP was determined in the presence of 24.7 μM acetyl-CoA and varying malonyl-ecACP concentrations (0.35−35 μM). All reaction components were incubated at 30 °C for 5 min, followed by addition of pfKASIII. Reactions were terminated during the linear phase (2 min) and analyzed using the protocol described above. Nonlinear regression with GraFit 4.012 (Middlessex, U.K.) was used to determine k_{cat} and K_M values.

Substrate Specificity of pfKASIII. To determine the acyl-CoA substrate specificity, radiolabeled acetyl-CoA, butyryl-CoA, and isobutyryl-CoA were used in a standard KAS assay. The 20 μL reaction consisted of the following components: 100 mM Na phosphate, pH 6.6, 2 mM 2-mercaptoethanol, 12 ng of pfKASIII, and 37 μM acyl-CoA (either [1-^{14}C]acetyl-CoA, [1-^{14}C]butyryl-CoA, or [1-^{14}C]-isobutyryl-CoA) in the presence of 11.1 μM malonyl-pfACP. The assays for determining ACP preference of pfKASIII were performed under the same conditions except that the concentration of [1-^{14}C]acetyl-CoA was maintained at 25 μM, while 10 μM of the ACP substrate (either malonyl-pfACP, malonyl-ecACP, pfACP, or ecACP) was used. These assays were initiated by addition of pfKASIII and incubated for 10 min at 30 °C. The [1-^{14}C]isobutyryl-CoA and malonyl-ecACP used in pfKASIII assays were prepared and purified as previously described (*27*).

Inhibition Studies. In vitro inhibition of pfKASIII activity was evaluated using TLM (0.50−333 μM), HR12 (0.0378−23.6 μM), HR19 (0.14−70 μM), and HR45 (0.0034−10.7 μM). In all inhibitor analysis, the inhibitors were first dissolved in dimethyl sulfoxide (DMSO) and then diluted to the required concentration followed by incubation with pfKASIII for 15 min at room temperature (23 °C). All assays, including controls, were carried out in 1% DMSO. The reaction was initiated by the addition of substrates malonyl-pfACP and [1-^{14}C]acetyl-CoA under the standard assay conditions described above. The concentrations of malonyl-pfACP (5.6 μM) and [1-^{14}C]acetyl-CoA (25 μM) used in these assays was comparable to the K_M values for these substrates. A control experiment in which pfKASIII was preincubated with 1% DMSO for 15 min in the absence of inhibitors was used to measure background in these experiments. The program GraFit 4.012 was used to calculate IC_{50} values.

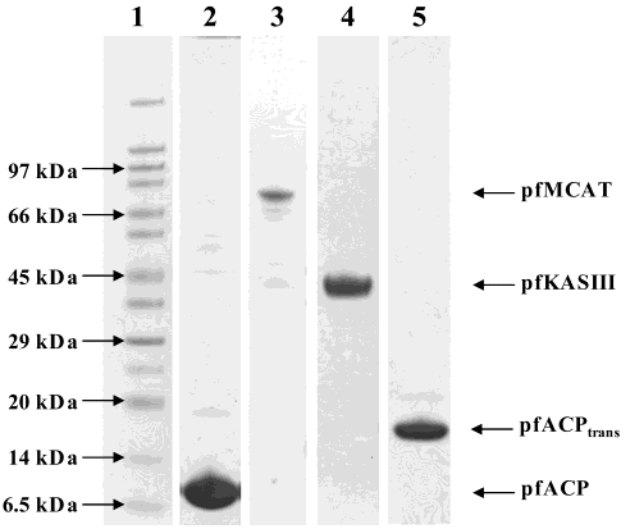

FIGURE 2: SDS−PAGE of pfACP, pfMCAT pfKASIII, and pfACP$_{trans}$. Lane 1 shows SigmaMarker wide range molecular weight standards (Sigma). Lanes 2−5 show pure recombinant pfACP (lane 2), pfMCAT (lane 3), pfKASIII (lane 4), and pfACP$_{trans}$ (lane 5). All proteins were cleaved with protease to remove the maltose binding protein tag except for pfMCAT which was purified as a fusion protein.

In Vitro Drug Susceptibility Assay. Compounds that inhibited pfKASIII (IC_{50} values below 10 μM) were tested in a cell-based in vitro drug susceptibility assay to determine if they were capable of interrupting *Plasmodium falciparum* metabolism and growth. The semiautomated microdilution technique of Desjardins et al. (*28*) was used to assess the sensitivity of the parasites to the selected compounds. The incorporation of [^{3}H]hypoxanthine into the parasites was measured as a function of compound concentration to determine IC_{50} values.

RESULTS

Expression and Purification of pfACP. In this study, we produced a construct of pfACP containing residues 57−137 and lacking the amino-terminal leader peptide. We expressed pfACP with an amino-terminal maltose binding protein fusion and purified it through affinity chromatography. The pure fusion protein was digested with factor Xa protease and repurified yielding cleaved pfACP without any affinity tag (Figure 2). This material had a low level of β-ketoacyl-ACP synthase (KAS) activity, presumably due to copurified *E. coli* KASIII. Filtration through a 30 kDa centrifuge concen-

FIGURE 3: Sequence alignment of *E. coli* and *P. falciparum* MCAT. The GXSXG consensus sequence common to various acylhydrolases is shaded. The amino-terminal residue of the truncated pfMCAT construct is indicated with an arrow. Identical residues are indicated with an asterisk and similar residues by a period.

trator removed the KAS activity from the pfACP sample. We analyzed pure pfACP using LC-MS (coupled liquid chromatography mass spectrometry) to determine how much pfACP was converted into holo-pfACP by *E. coli* holo-ACP Synthase (AcpS) through incorporation of a 4′- phosphopantetheine prosthetic group (339 Da). We observed almost complete conversion to holo-pfACP and were unable to detect any apo-pfACP by LC-MS (data not shown). Interestingly, LC-MS analysis showed that about 10% of the holo-ACP was acylated (C12:0 and C14:0 were observed).

We also produced a construct of pfACP containing residues 17−137 and including the putative apicoplast transit peptide. This construct ($pfACP_{trans}$) was designed to mimic pfACP as it exists in the secretory space—after processing of the signal peptide and prior to import into the apicoplast and cleavage of the transit peptide. LC-MS analyses revealed once again that this protein was in the holo form. Minor components (<10%) of the holo-$pfACP_{trans}$ were observed to be acylated with C:12, C:14 and C:16 saturated fatty acids. It has been suggested in the past that formation of acylated ACP species indicates that the heterologous ACP serves as a substrate for *E. coli* enzymes involved in Type II fatty acid biosynthesis (*29*, *30*).

Expression and Purification of pfMCAT. We used genomic sequences found in PlasmoDB (*31*) to identify the *pfMCAT* gene and to design primers for PCR amplification from cDNA. Two closely spaced start codons (encoding M1 and M6) appeared equally likely to be the amino-terminus of the protein. We based our primer design on the first start codon and amplified the *pfMCAT* gene from *P. falciparum* cDNA. We verified the sequence of full length *pfMCAT* and found that it was identical to the genomic sequence found in PlasmoDB (*31*). The amino-terminal 103 amino acids of pfMCAT were not homologous to bacterial MCAT enzymes and appeared to form a leader peptide (Figure 3). Residues 1−31 are hydrophobic and are strongly predicted to be a signal peptide by the SignalP algorithm (*32*). The next 22 residues are acidic (F32 to N53; pI = 6.2), followed by a 20 residue basic region (K54 to K73; pI = 10.7) and a 30 residue acidic region (G74 to Y103; pI = 5.7). The pfMCAT without the putative leader peptide is only 28% identical to *E. coli* MCAT (FabD) and contains a GXSXG consensus sequence (*33*) common to various acylhydrolases (Figure 3). For our functional studies, we designed a construct encoding residues 104−408 and lacking the putative leader peptide. This fragment could be expressed in a soluble form as a fusion protein with the maltose binding protein (Figure 2), but could not be efficiently cleaved from the affinity tag and repurified. Efforts to express the pfMCAT enzyme without the maltose binding protein resulted in high yields of insoluble protein.

MCAT Activity and Generation of Malonyl-pfACP. We used [1-^{14}C]-malonyl-CoA to assay the malonyl-coenzyme A:ACP transacylase (MCAT) activity of pfMCAT. Under the conditions described in the methods section, we were able to observe 36% conversion of pfACP to malonyl-pfACP in a 30 min period at 37 °C. No detectable conversion of the $pfACP_{trans}$ was observed under these conditions using pfMCAT. As noted above, $pfACP_{trans}$ was shown by LC-MS to be in the holo form, and thus the lack of activity was not due to the presence of the apo form. Formation of sufficient amounts of malonyl-pfACP in addition to malonyl-ecACP was critical to the study, as it not only provided substrates to test the β-ketoacyl-ACP synthase activity of pfKASIII, but also to probe the ACP specificity of the enzyme.

Expression and Purification of pfKASIII. We produced a construct of pfKASIII containing residues 50−371 and lacking the amino-terminal leader peptide. We expressed

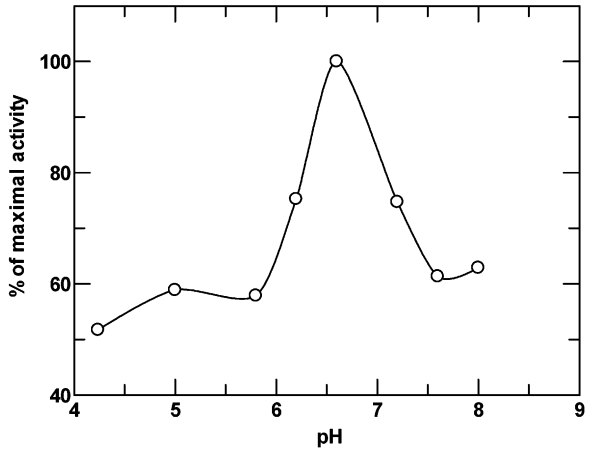

FIGURE 4: A pH profile of the β-ketoacyl-ACP synthase actitivy of *P. falciparum* KASIII.

Table 1: Kinetic Parameters of *P. falciparum* KASIII Activity

substrate	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}\,\mu M^{-1}$)
acetyl-CoA	17.9 ± 3.4	230	12.8
butyryl-CoA	35.7 ± 4.4	199.7	5.6
malonyl-pfACP	5.2 ± 0.5	336.0	64.9
malonyl-ecACP	3.0 ± 0.7	135.3	44.8

pfKASIII with an amino-terminal maltose binding protein fusion to increase the yield of expressed protein. The linker region of this construct contained a tobacco etch virus (TEV) protease recognition site followed by a six histidine tag amino-terminal to the pfKASIII domain. In vivo cleavage of the fusion protein by coexpressed TEV protease released the pfKASIII domain with an amino-terminal histidine tag. The cleaved protein lacking the maltose binding protein was then purified by metal chelate and cation exchange chromatography (Figure 2).

KAS Activity. Successful optimization of the pfMCAT assay allowed us to produce enough malonyl-pfACP to assay the β-ketoacyl-ACP synthase (KAS) activity of pfKASIII. Under the conditions described in the methods section, we were able to observe the formation of acetoacetyl-pfACP using malonyl-pfACP and [1-^{14}C]acetyl-CoA as substrates. The activity of pfKASIII was measured using these substrates over the range from pH 4.2 to 8.0 using standard assay conditions. The activity of pfKASIII showed a significant pH dependence with maximal activity at pH 6.6 (Figure 4).

Enzyme Kinetics of pfKASIII. We observed typical saturation kinetics with pfKASIII in response to increasing concentrations of acyl-CoA substrates. Apparent K_M values of 17.9 ± 3.4 μM for acetyl-CoA and 35.7 ± 4.4 μM for butyryl-CoA were determined (Table 1 and Figure 5A,B). The affinity of acetyl-CoA for pfKASIII (17.9 μM) is similar to that determined for ecKASIII (40 μM) (*34*) and *S. pneumoniae* KASIII (40.3 μM) (*35*) and higher than that observed for the *S. glaucescens* KASIII (2.4 μM) (*27*) and *S. aureus* KASIII (6 μM) (*36*). Similarly, the K_M value for butyryl-CoA (35.7 μM) is much higher than in *S. glaucescens* KASIII (0.6 μM) and *S. aureus* KASIII (2.32 μM) (*27, 36*). The k_{cat} using acetyl-CoA was 230 min^{-1}, almost 6-fold higher than that recently reported for *S. glaucescens* KASIII (42 min^{-1} using acetyl-CoA and the *S. glaucescens* ACP as

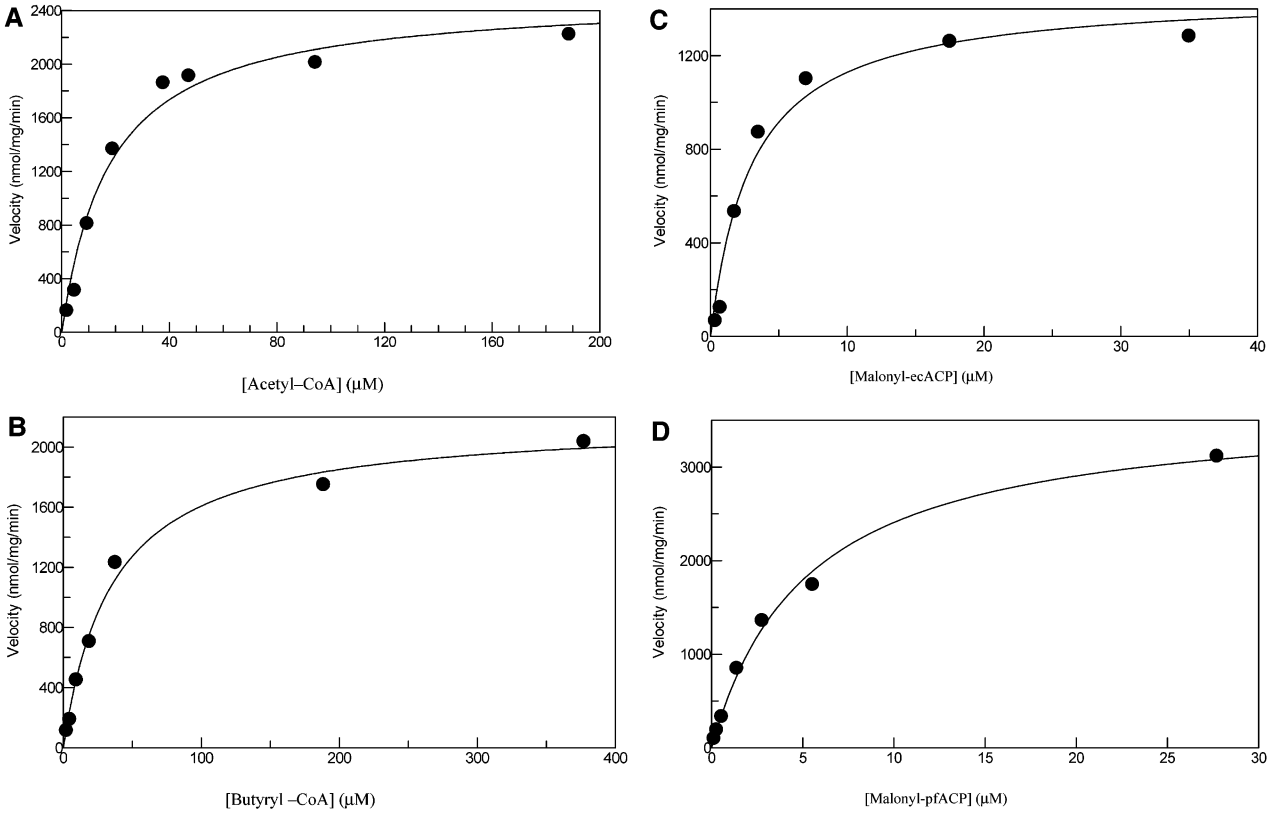

FIGURE 5: Kinetic analysis of *P. falciparum* KASIII with acyl-CoA and malonyl-ACP substrates. The initial velocities of product formation were measured with pfKASIII and malonyl-pfACP in the presence of increasing concentrations of acetyl-CoA (A) and butyryl-CoA (B). The initial velocities of product formation were measured with pfKASIII and acetyl-CoA in the presence of increasing concentrations of malonyl-ecACP (C) and malonyl-pfACP (D).

Table 2: Substrate Specificity of *P. falciparum* KASIII[a]

substrate	enzyme activity (nmoles min^{-1} μg^{-1})
acetyl-CoA	0.85 ± 0.08
butyryl-CoA	0.62 ± 0.01
isobutyryl-CoA	0.064 ± 0.009
pfACP	<0.001
malonyl-pfACP	0.69 ± 0.01
ecACP	<0.001
malonyl-ecACP	0.25 ± 0.05

[a] Specific activity was determined using the assay conditions described in the methods section. Values represent the average of duplicated assays.

substrates) (*27*). A comparison of the catalytic efficiency (k_{cat}/K_M) of pfKASIII using acetyl-CoA (12.8 $min^{-1}\mu M^{-1}$) versus butyryl-CoA (5.6 $min^{-1}\mu M^{-1}$) revealed a slight preference for acetyl-CoA (Table 1). Saturation kinetics were also observed with pfKASIII in response to increasing concentrations of malonyl-pfACP with an apparent K_M of 5.2 ± 0.5 μM (Table 1 and Figure 5C,D). A similar K_M was found using malonyl-ecACP (3.0 ± 0.7 μM), indicating that pfKASIII is unable to distinguish significantly between *E. coli* and *P. falciparum* malonyl-ACP (Table 1 and Figure 5).

Substrate Specificity of pfKASIII. We tested the acyl-CoA specificity of pfKASIII using radiolabeled acetyl-CoA, butyryl-CoA and isobutyryl-CoA. Isobutyryl-CoA is not a good substrate for pfKASIII and exhibited 13-fold less activity (0.064 ± 0.009 nmol min^{-1} μg^{-1}) than that observed with acetyl-CoA (0.848 ± 0.08 nmol min^{-1} μg^{-1}) under the same assay conditions (Table 2). Poor reactivity with isobutyryl-CoA indicates that pfKASIII is probably not involved in the synthesis of branched chain fatty acids as found in some bacterial systems. Other than its major KAS activity, a minor acetyl-CoA:ACP transacylase (ACAT) activity has been reported in KASIII enzymes from several sources, typically ranging from 1% to 10% of KAS activity (*19*, *27*). To study the ratio of ACAT to KAS activity in pfKASIII, the enzyme was assayed in the presence of [1-^{14}C]-acetyl-CoA using either pfACP or malonyl-pfACP as substrates. The KAS activity of the pfKASIII was greater than the ACAT activity by a factor of greater than 690 (Table 2). A similar KAS:ACAT activity ratio was observed for the pfKASIII using ecACP and malonyl-ecACP as substrates (Table 2).

Inhibition Studies. Thiolactomycin is an inhibitor of bacterial condensing enzymes and has previously been evaluated for antimalarial activity (*5*). Thiolactomycin was shown to inhibit the growth of malaria parasites in culture (IC_{50} of 50 μM), and this antimalarial activity was attributed to inhibition of the FAS condensing enzymes (*5*). We tested

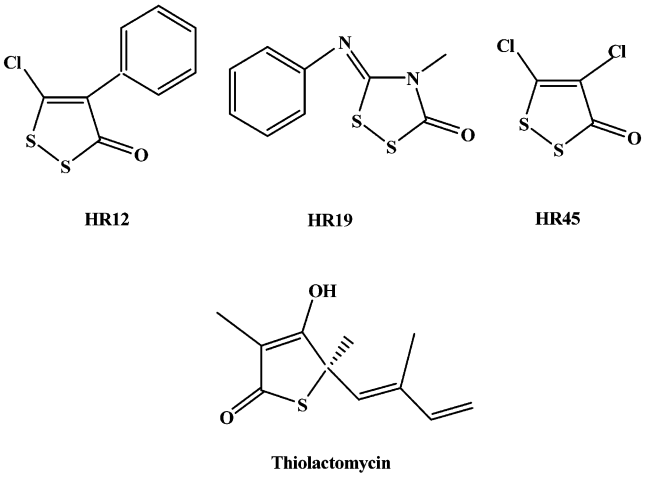

FIGURE 6: Structures of inhibitors tested in this study.

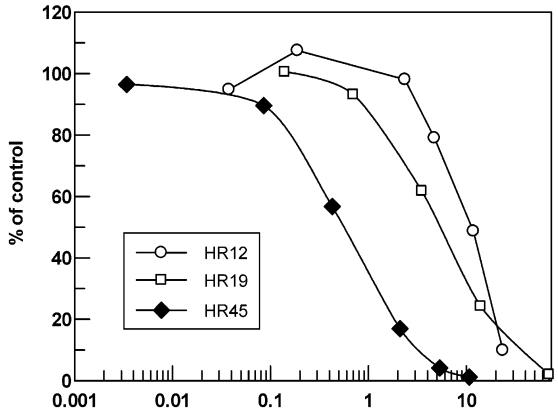

FIGURE 7: Inhibition of *P. falciparum* KASIII by compounds HR12, HR19, and HR45. Inhibitor structures are shown in Figure 6.

thiolactomycin and found it to be a poor inhibitor (IC_{50} > 330 μM) of pfKASIII (Table 3). In an effort to identify better inhibitors, we tested several structurally similar compounds from the National Cancer Institute database. Three of these compounds inhibited pfKASIII with IC_{50} values at or below 10 μM (Figure 6 and Figure 7). These compounds also inhibited the growth of malaria parasites in culture when tested against the chloroquine-sensitive D6 strain and the chloroquine-resistant W2 strain of *Plasmodium falciparum* (Table 3).

DISCUSSION

Three proteins are necessary to initiate fatty acid biosynthesis in a Type II FAS system: ACP, MCAT, and KASIII. Previous work indicated that pfACP and pfKASIII are targeted to the apicoplast organelle of the malaria parasite with a bipartite leader peptide (*5*, *6*, *22*). The leader peptides

Table 3: *P. falciparum* KASIII Inhibition and *P. Falciparum* Drug Susceptibility

compound	NCI #	CAS #	pfKASIII IC_{50} (μM)	D6 *P. falciparum* drug susceptibility IC_{50} (μM)	W2 *P. falciparum* drug susceptibility IC_{50} (μM)
TLM		82079-32-1	>330	nd[a]	50[b]
HR12	29804	2425-05-0	10.4 ± 1.04	8.3	12.2
HR19	3415	5338-82-9	5.31 ± 0.24	8.7	9.4
HR45	135976	1192-52-5	0.53 ± 0.03	27	45

[a] Not determined. [b] Data from ref *5*.

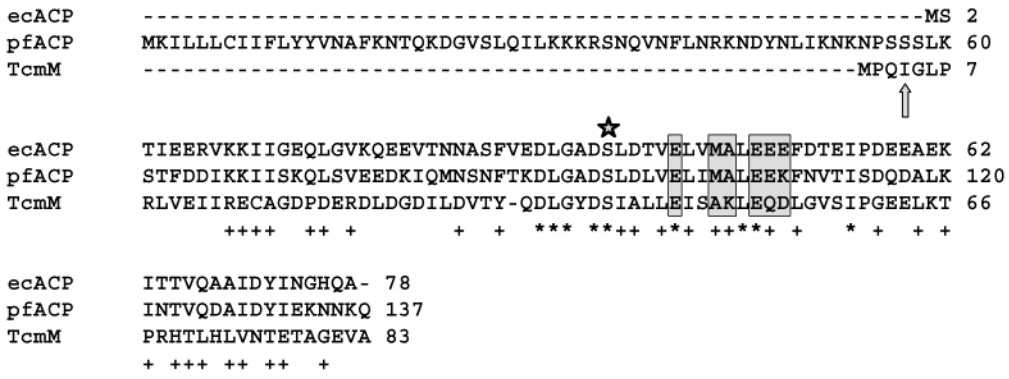

FIGURE 8: Multiple sequence alignment of *E. coli*, *P. falciparum*, and TcmM ACP. Residues predicted to be important in forming an interaction between the *E. coli* KASIII and ACP (*46*) are shaded. The conserved serine required for attachment of the 4′-phosphopantetheine group is marked with a star. The amino-terminal residue of the truncated pfACP construct is indicated with an arrow. Identical residues are indicated with an asterisk, and residues identical between ecACP and pfACP are marked with a plus sign.

of pfACP and pfKASIII consist of a hydrophobic signal peptide for entry into the secretory pathway followed by a basic (pI > 10) transit peptide about 24 amino acid in length for subsequent import into the apicoplast. The pfMCAT identified in this study also appears to possess a leader peptide, but it is 103 amino acids in length and displays a more complicated architecture. Residues 1−31 are predicted to be a hydrophobic signal peptide by the SignalP algorithm (*32*), but the following 22 amino acid peptide is acidic (pI = 6.21) and does not seem suited for apicoplast transit (*37*). This acidic region is followed by a basic (pI = 10.7) region and another acidic region (pI = 5.7). The basic region is perhaps responsible for apicoplast targeting. Consistent with our expectations, truncated pfMCAT lacking the leader peptide was active using pfACP as substrate, suggesting a physiological role in fatty acid biosynthesis within the apicoplast organelle. We found that a pfACP construct containing the apicoplast transit peptide ($pfACP_{trans}$) was not a substrate for pfMCAT even though LC-MS showed that $pfACP_{trans}$ was produced entirely in the holo form. This result suggests that malonyl-ACP will only be formed in the lumen of the apicoplast after cleavage of the transit peptide from ACP. Since Type II FAS systems require malonyl-ACP, it seems likely that fatty acid biosynthesis will not occur outside the apicoplast organelle of the malaria parasite.

To be a substrate for either the pfMCAT or pfKASIII, the pfACP must be converted from the apo-form to the holo form. This step involves transfer of a 4′-phosphopantetheine moiety from coenzyme A to a conserved serine residue on the ACP (Ser95). In *E. coli* this transfer is catalyzed by a 126 amino acid enzyme called holo-ACP synthase (AcpS) (*38*). An AcpS presumably must also function in *P. falciparum* to generate holo-pfACP. Analysis of the *P. falciparum* genome (*31*) revealed a candidate gene on chromosome 4 that codes for a protein that is 29% identical to *E. coli* AcpS. The putative pfAcpS appears to have an appropriate leader peptide for apicoplast targeting; however, it is difficult to assign the amino-terminus of this protein because the AcpS homology domain is preceded by a large open reading frame (450 amino acids in length). The amino-terminal region of pfAcpS, including possible processing of the leader peptide, may affect AcpS activity since mutations at the amino-terminus of the *B. subtilis* AcpS have been shown to radically affect the ability of the enzyme to form the active trimer (*39*). Thus, it seems possible that the pfAcpS is targeted to the apicoplast where it catalyzes conversion of apo-pfACP to holo-pfACP.

The recombinant pfACP obtained in this study was predominantly in the holo-form, suggesting that it was readily modified by the *E. coli* AcpS. This enzyme has broad substrate specificity and can modify heterologous ACPs, such as those from bacteria (*Streptomyces glaucescens* and *Mycobacterium tuberculosis*) and plants (spinach), when they are expressed in *E. coli* (*29*, *40*, *41*). ACPs involved in different processes, such as polyketide biosynthesis, are poorer substrates and are often obtained predominantly in the apo-form when expressed alone (without overexpression of AcpS) in *E. coli* (*40*, *42*). The pfACP (expressed with a 43 kDa maltose binding protein at the amino-terminus) and $pfACP_{trans}$ (expressed with the amino-terminal transit peptide) were obtained almost exclusively in the holo form, suggesting that the amino-terminal region of ACPs does not play a significant role in substrate recognition by AcpS. These observations are consistent with a recent crystallographic study of the *Bacillus subtilis* AcpS−ACP complex which shows that the major interactions occur along an α helix of ACP that is far removed from the amino-terminal region (*39*). Pairwise sequence alignment of pfACP and ecACP shows they are highly similar in the region corresponding to this helix, while a recent analysis of this region for TcmM, a polyketide synthase ACP (*40*), showed significant differences (Figure 8).

The acyl group specificity of KASIII enzymes is a determining factor in the type of fatty acids produced by a Type II FAS (*43*). The bacterial KASIII enzymes (FabH) from *S. glaucescens*, *B. subtilis*, and *S. aureus* have relaxed substrate specificities and use a variety of straight-chain and branched-chain acyl-CoA substrates (*27*, *36*, *43*). This relaxed specificity is consistent with the ability of these bacteria to synthesize both straight-chain and branched-chain fatty acids (*43*). In contrast, the KASIII enzymes from *E. coli* and *S. pneumoniae*, organisms which generate straight chain fatty acids exclusively (*44*) discriminate against branched-chain acyl-CoA substrates such as isobutyryl-CoA. Poor reactivity with isobutyryl-CoA relative to acetyl-CoA (Table 2) indicates that pfKASIII is not involved in the synthesis of branched-chain fatty acids and that *P. falciparum* makes straight-chain fatty acids. Butyryl-CoA is a good substrate for pfKASIII (Table 1 and Table 2) but not the *E. coli* KASIII, suggesting that the malaria enzyme could use short

straight-chain acyl-CoA substrates other than acetyl-CoA (it is not clear whether this activity is physiologically relevant since little is known about the pools of acyl-CoA substrates in the apicoplast organelle of the malaria parasite). In the structure of *E. coli* KASIII, five hydrophobic residues (Leu142, Phe157, Leu189, Leu205, and Phe87) form a substrate binding pocket for acyl-CoAs. This pocket was proposed to be responsible for restricting ecKASIII to either acetyl-CoA or propionyl-CoA as substrates, but not butyryl-CoA (*45*). In pfKASIII the binding pocket residues are conserved despite the ability of butyryl-CoA to be a substrate. Similarly, recent analysis of the *S. aureus* KASIII has shown these residues are also conserved, despite the enzyme being able to use larger acyl substrates such as isobutyryl-CoA and butyryl-CoA (*36*). Thus, conservation of these residues does not allow for accurate prediction of KASIII substrate specificity. It is curious that the reported K_M values for acetyl-CoA are higher (18−40 μM) for KASIII enzymes (*P. falciparum*, *S. pneumoniae* and *E. coli*) initiating straight chain fatty acid biosynthesis than values for KASIII enzymes (*S. aureus*, 6 μM and *S. glaucescens*, 2.5 μM) initiating both straight-chain and branched-chain fatty acid biosynthesis (*27*, *34*−*36*). The structural basis and physiological relevance of this observation, made from a very limited data set, are not apparent.

E. coli malonyl-ACP (malonyl-ecACP) appeared to be comparable to malonyl-pfACP as a substrate for pfKASIII (Table 1). It is possible that contaminating pfACP in our malonyl-pfACP samples acted as a competitive inhibitor in the KAS assays, masking a true preference for malonyl-pfACP. However, no substantial differentiation between the two ACPs was observed in the acetyl-CoA:ACP transacylase (ACAT) activity of pfKASIII either (Table 2). This lack of significant ACP specificity may not be too surprising since *E. coli* and *P. falciparum* ACP share 50% sequence identity. Perhaps more important is the conservation of residues in the α helix which is likely involved in binding to AcpS (Figure 8). A recent docking model describing the interaction of ecKASIII and its cognate ecACP indicates that the acidic amino acids in this helix are also important for binding to basic residues in the ecKASIII active site (*46*). Five out of the six residues identified in this docking study (ecACP residues Glu42, Met45, Ala46, Glu48, Glu49, and Glu50) are conserved in pfACP, making it possible that the binding mode of ACP is conserved between the bacterial and malarial systems (Figure 8). The only difference is residue Glu50 of ecACP which is basic (Lys108) in pfACP (presumably this residue does not play a critical role in the interaction of ACPs with the pfKASIII). In contrast, these residues are less well conserved in TcmM (Figure 8), a polyketide synthase ACP that has recently been shown to be a poor substrate for KASIII enzymes (*40*).

It is unclear if there is any physiological relevance to the observation that KASIII enzymes possess ACAT activity. The acetyl-ACP product of ACAT activity may be a substrate of other condensing enzymes (*47*). However, it is unlikely that a significant amount of acetyl-ACP accumulates since ACAT activity is typically very low compared to KAS activity (1% for the *E. coli* KASIII (*19*)). A higher level of ACAT-to-KAS activity (12%) was reported for the *S. glaucescens* KASIII using *E. coli* ACP as substrate (*27*). However, recent analyses show that the KAS activity of this enzyme is increased dramatically (6-fold) when the cognate ACP from *S. glaucescens* (FabC) is used (*40*). When the physiological ACP is used, the ratio of ACAT to KAS activity for *S. glaucescens* KASIII may be similar to that seen in the *E. coli* system. In pfKASIII, the ACAT activity is exceptionally low—less than 0.15% of the KAS activity (Table 2). Some degree of ACAT activity is perhaps an unavoidable consequence for an enzyme which generates an acylated enzyme intermediate from acetyl-CoA and has an ACP binding site. Indeed, it has recently been observed that a thiolase from *S. collinus* which generates a similar acylated enzyme intermediate has a low level of ACAT activity (<0.2% of thiolase activity) even though this enzyme does not use ACP as a physiological substrate and there is presumably no ACP binding site (*48*).

Thiolactomycin (TLM) was shown in this study to be a poor inhibitor of pfKASIII with an IC_{50} value greater than 330 μM (Table 3). Waller et al. showed that TLM inhibits the proliferation of cultured *P. falciparum* with an IC_{50} value of 50 μM and attributed this antimalarial activity to inhibition of the FAS condensing enzymes (*5*). It is likely that TLM targets the second condensing enzyme in *P. falciparum* (a gene encoding a putative pfKASI/II is located on chromosome 6 (*31*)) rather than pfKASIII. While TLM is an effective inhibitor of some bacterial KASIII enzymes (*35*), it is significantly more effective against *E. coli* KASI (FabB) rather than *E. coli* KASIII (FabH) (*49*). We tested several 1,2-dithiole-3-ones which bear structural similarity to TLM and found three that inhibited pfKASIII with IC_{50} values at or below 10 μM (Figure 6 and Figure 7). Two of these inhibitors, 5-chloro-4-phenyl-[1,2]-dithiole-3-one (HR12) and 4-phenyl-5-phenylimino-[1,2,4]-dithiazolidin-3-one (HR19), have recently been shown to be active against the *S. aureus* FabH, suggesting that they may be general KASIII inhibitors (the mechanism of inhibition by these compounds is currently under investigation). These compounds also inhibit the growth of *S. aureus* (*36*) and malaria parasites in culture (Table 3), demonstrating that these compounds cross the cell wall readily, regardless of their cellular target. In *S. aureus* the biochemical basis for this antibacterial activity of HR12 remains unknown, although preliminary investigations do not indicate a specific inhibition of fatty acid biosynthesis (*50*).

In conclusion, we have demonstrated β-ketoacyl-ACP synthase (KAS) activity using pure recombinant pfACP, pfMCAT, and pfKASIII lacking their amino-terminal leader peptides. The acyl group and ACP specificity of the pfKASIII enzyme have also been investigated. These results establish that malaria parasites can catalyze the initiating steps of a Type II FAS in the apicoplast organelle of the malaria parasite. Moreover, the failure of $pfACP_{trans}$ (pfACP with apicoplast transit peptide) to be a substrate of pfMCAT suggests that fatty acid biosynthesis will only occur in the lumen of the apicoplast. The identification of pfKASIII inhibitors able to kill malaria parasites in culture may prove to be a valuable tool in designing new strategies to combat malaria.

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