The human malaria parasite *Plasmodium falciparum* synthesizes fatty acids by using a type II synthase that is structurally different from the type I system found in eukaryotes. Because of this difference and the vital role of fatty acids, the enzymes involved in fatty acid biosynthesis of *P. falciparum* represent interesting targets for the development of new antimalarial drugs. β-Ketoacyl-acyl carrier protein (ACP) synthase (PfFabBF), being the only elongating toacyl-ACP synthase in *P. falciparum* and the vital role of fatty acids, the enzymes involved in fatty acid biosynthesis by using a type II synthase that is structurally different from the type I system found in eukaryotes. Because of this difference and the vital role of fatty acids, the enzymes involved in fatty acid biosynthesis of *P. falciparum* represent an important step toward the evaluation of the Unique Elongating ACP for binding (16–21).

Using Natural and Artificial Substrates*

The recent completion of the genome sequencing of *P. falciparum* allowed the identification of highly promising pathways in the parasite (4). One of the most interesting discoveries was the presence of a complete type II fatty acid biosynthesis pathway (FAS2-II) (5, 6). FAS-II is found in bacteria and plants and is structurally very different from the FAS-I system found in most eukaryotes. In FAS-I, the biosynthetic enzymes are integrated into a large multifunctional single polypeptide (7), whereas FAS-II uses separate, discrete enzymes that carry out the individual steps during initiation and chain elongation (8).

The importance of lipids for parasite survival has generated much interest in the enzymes responsible for their biosynthesis. Their inhibition has been shown repeatedly to be a suitable target for antimicrobials (9). Thus, the intervention at the level of *P. falciparum* FAS-II represents a very promising approach for the development of new antimalarials (10, 11).

Among the inhibitors described to act against various targets in FAS-I and FAS-II, thiolactomycin and cerulenin are known to inhibit the β-ketoacyl-ACP synthases FabB and FabF of bacteria (e.g. *Escherichia coli* and *Mycobacterium tuberculosis*) and plants (e.g. *Pismum sativum* and *Allium porrum*) (12–17). Cerulenin inactivates the enzymes irreversibly, forming a covalent adduct with the active site cysteine (14). Thiolactomycin is a reversible inhibitor that competes with malonyl-ACP for binding (16–21). β-Ketoacyl-ACP synthases catalyze the Claisen condensation reaction, transferring an acyl primer to malonyl-ACP and thereby creating a β-ketoacyl-ACP product that has been lengthened by a two-carbon unit. In the type II FAS of plants and bacteria, three β-ketoacyl-ACP synthases with different substrate specificities have emerged as important regulators of the initiation and elongation steps in the pathway. The initiation enzyme β-ketoacyl synthase III (FabH) only catalyzes the elongation of malonyl-ACP by an acetyl-CoA primer, whereas the elongation enzymes β-ketoacyl synthase I and II (FabB and FabF) use acyl-ACPs for elongation of malonyl-ACP (18).

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2 The abbreviations used are: FAS, fatty-acid synthase; PFACP, *P. falciparum* acyl carrier protein; EcACP5, *E. coli* holo-acyl carrier protein synthase; PfFabBF, *P. falciparum* β-ketoacyl-ACP synthase PfFabBF, *P. falciparum* β-ketoacyl-ACP reductase; DTT, dithiothreitol; CER, cerulenin; TLM, thiolactomycin; TB, terrific broth; CS-PAGE, conformational sensitive gel electrophoresis; BisTris, 2-[bis(2-hydroxy-ethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

Malaria is one of the world’s most important infectious diseases in terms of both mortality and morbidity. The consensus is that 0.5 billion clinical attacks take place every year, including 2–3 million severe attacks (1–3). It is assumed that the disease claims more than 1 million lives annually and that most of these deaths occur in African children, but the true number might be much higher. Chloroquine, which used to be the first line treatment for malaria, now fails everywhere. Emerging resistance to drugs introduced to replace chloroquine, such as sulfadoxine-pyrimethamine, reinforces the need for new, selective, and affordable drugs against the parasite (1). Of the four causative agents of malaria, i.e. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*, *P. falciparum* is the most dangerous. The recent completion of the genome sequencing of *P. falciparum* allowed the identification of highly promising pathways in the parasite (4). One of the most interesting discoveries was the presence of a complete type II fatty acid biosynthesis pathway (FAS2-II) (5, 6). FAS-II is found in bacteria and plants and is structurally very different from the FAS-I system found in most eukaryotes. In FAS-I, the biosynthetic enzymes are integrated into a large multifunctional single polypeptide (7), whereas FAS-II uses separate, discrete enzymes that carry out the individual steps during initiation and chain elongation (8).
Sequenceing of the full genome of *P. falciparum* revealed that the parasite possesses only one isoform of the elongating condensing enzyme, PfFabBF. As PfFabBF is unique, its activity cannot be replaced by another β-ketoacyl-ACP synthase, which makes it a very promising target. To date there are no data available concerning substrate specificity and kinetic parameters of PfFabBF. To this end, we present the cloning, expression, and purification of this enzyme. A recombinant expression and purification system was established, allowing the production of significant amounts of active enzyme that are suitable for the characterization using natural acyl-PfACP substrates as well as the corresponding acyl-CoA analogs.

**EXPERIMENTAL PROCEDURES**

**Materials and Instrumentation**—All cloning steps were performed in *E. coli* Nova Blue cells (Stratagene). Expression was conducted in *E. coli* BL21(DE3)-CodonPlus-RIL cells (Stratagene). Sources of supplies are as follows: pET vectors from Novagen; Pf Turbo polymerase from Stratagene; T4 DNA ligase and restriction enzymes from New England Biolabs; plasmid extraction kits from Sigma; NADPH from Roche Diagnostics; Terrific broth (TB) medium from Difco; cerulenin and acyl-CoAs from Sigma; nickel-nitritriacetic acid-agarose from Qiagen; Hitrap, HisTrap, PD10 and Superdex 75/200 gel filtration columns from Amersham Biosciences. An Akta FPLC (Amersham Biosciences) was used for protein purification. Spectrophotometric measurements were carried out on a Cary 50 concentration Varian Spectrophotometer using an ultra-micro cell from Hellma (type 105.202-QS). All other supplies were reagent grade or better.

Multiple Sequence Alignment—Sequences of β-ketoacyl-ACP synthases of various organisms were aligned using the ClustalW alignment program (22). The sequences were obtained from the SwissProt data base and included *E. coli* FabB (SwissProt primary accession number P14926) and FabB (P39435), *Arabidopsis thaliana* FabB (P52410) and FabB (Q9C9P4), and *P. falciparum* FabBF (Q6LF11).

Cloning of the *P. falciparum* PfFabBF Expression Plasmids—A two-step megaprim PCR method (23, 24) was used to create a pfFabBF clone that carried a 171-bp truncation at the N terminus. In the first step, two overlapping fragments of the pfFabBF gene were PCR-amplified from *P. falciparum* 3D7 gDNA (MRA-386, MR4; ATCC, Manassas VA). The first fragment was amplified using the forward primer 5'-ACCTTCTGAAGTGTTGCTGCACAG and the reverse primer 5'-CCGAACTTCTCCTCCATAAAGAAACACACAG. The second fragment was generated using the following primer pair: 5'-GAAGTGGTTTCGTTATGGGAGAAGGTTCAGG and 5'-TCACCTTTTATTTTTTGAAGATAAG. In a second step, the above mentioned fragments were combined to give the final insert using forward primer 5'-TGGTTCCATGGGTTTTCTAAGTGTTGCTGCACAGGTAGGGTA and reverse primer 5'-CCGAACTTCTCCTCCATAAAGAAACACACAG.

An 18-bp-long stretch of DNA, coding for six additional amino acids (i.e. KNLCET) of the original pfFabBF N terminus, was introduced by site-directed mutagenesis using as template the plasmid described above. The following primers were used: 5'-GGTACCGACGACGACGACGACGACGACGACGACTGATAGAAGGCCAAACTTTTGTGGAACCTTCTAGAGTGGTGTGCACAGGTAGGGTA and 5'-ACCCTTACACTTGTGACAAC.

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**RESULTS**

**Construction of the PfFabBF expression vector.** pET30b-PfFabBF was digested with BglII and EcoRI, and the resulting fragment was subcloned into an equally digested pET42b vector. Cleavage with SpeI and EcoRI generated a fragment that contained the whole PfFabBF open reading frame, including a His tag as well as an S tag, which was inserted into a pET43.1b vector to give the final pET43.1b-PfFabBF expression plasmid.

**Expression and Purification of PfFabBF**—The final pET43.1b-PfFabBF clone was designed to express PfFabBF as NusA fusion protein with an expected molecular mass of 107 kDa, covering amino acids 52–474 of the β-ketoacyl-ACP synthase part. The expression plasmid was introduced into BL21(DE3)-CodonPlus-RIL expression cells. The cells were used to inoculate 500 ml of TB medium supplemented with 1
**β-Ketoacyl-ACP Synthase from *P. falciparum***

mm betaine, 660 mm sorbitol, ampicillin (100 μg/ml), and chloramphenicol (34 μg/ml) and grown overnight at 37 °C. The temperature was then lowered to 25 °C, and protein production was induced with 1 mm isopropryl β-D-thiogalactopyranoside for 7 h. The cells were harvested by centrifugation, resuspended in lysis buffer (20 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole, 2 mM DTT, 10% glycerol, pH 7.5) containing DNase, and disrupted by passing them twice through a French press. The extract was clarified, and the supernatant was applied to a 5-ml HisTrap chelating column. The column was washed with 4 column volumes of lysis buffer and eluted by means of a 50-ml linear imidazole gradient using buffer A (20 mM NaH₂PO₄, 500 mM NaCl, 2 mM DTT, 10% glycerol, pH 7.5) and buffer B (20 mM NaH₂PO₄, 500 mM NaCl, 500 mM imidazole, 2 mM DTT, 10% glycerol, pH 7.5). Fractions containing PfFabBF were pooled, concentrated, (Centriprep, 30-kDa cut-off), diluted to a final NaCl concentration of 50 mM, and loaded on a 5-ml HiTrap SP-Sepharose HP column. The protein was eluted with a linear NaCl gradient using buffer C (20 mM NaH₂PO₄, 2 mM DTT, pH 7.5) and buffer D (20 mM NaH₂PO₄, 1 mM NaCl, 2 mM DTT, pH 7.5). The fractions containing the fusion protein were again pooled, concentrated, and either applied on a Superdex 200 column equilibrated with buffer E (20 mM NaH₂PO₄, 300 mM NaCl, 2 mM DTT, 10% glycerol, pH 7.5) or desalted by ultrafiltration (Amicon Ultra 4, 30-kDa cut-off), depending on the required degree of purity. Total protein concentration was determined by a dye-binding assay (25). The purified protein was stored at +4 °C.

Cloning and Expression of *P. falciparum* ACP—Acyl-PCAPs are the natural substrates of PfFabBF and are thus needed for its characterization. PfACP has been cloned and expressed in a similar way as described earlier (26, 27). Briefly, the pfacp gene sequence N-terminally truncated by 180 bp was PCR-amplified from a *P. falciparum* strain 3D7 gametocyte stage cDNA pSPORT plasmid library (kindly provided by Dr. T. Templeton, Weil Medical College of Cornell University) and ligated into the PET28b expression vector. Correct clones were identified and verified by restriction digestion, PCR, and automated sequencing with T7 forward and reverse primers. The resulting clone was designed to express the enzyme without the putative N-terminal signal and translocation sequence, i.e. amino acids 1–54. PfFabG, consisting of residues 55–304, was expressed for 6 h at 37 °C using BL21(DE3)-CodonPlus-RIL cells. Purification of the protein was performed as described for PfFabI (31), except that a linear imidazole gradient was applied.

Preparation of Acylated PfACP Substrates—C₄₀ to C₁₆₀ acyl-PCAPs were generated using EcACPS. A typical reaction (1 ml) contained 100 μM of the corresponding acyl-CoA, 250 μg of EcACPS, 1 mg of apo-PfACP, 25 mM MgCl₂ in 20 mM Tris, pH 7.5. For higher amounts of substrates, the protocol was scaled up accordingly. The mixture was incubated at 37 °C for 1 h. EcACPS was eliminated from the mixture by binding it to nickel-nitrioltriacetic acid-agarose. Afterward the acyl-PCAPs were concentrated and desalted by means of a PD10 column equilibrated with 20 mM NaH₂PO₄ and 300 mM NaCl, pH 7.5. The purity of acyl-PCAPs was confirmed by CS-PAGE (28, 29).

Enzyme Assays—A continuous assay format was used to monitor the PfFabBF activity by coupling the condensing activity of PfFabBF to PfFabG. FabG reduces β-ketoacyl-ACPs to the corresponding β-hydroxyacyl-ACPs by simultaneous oxidation of its cofactor NADPH to NADP⁺, allowing the reaction course to be monitored spectrophotometrically at 340 nm. Control experiments, such as performing the reaction either without substrates or without enzyme, were carried out to rule out unspecific oxidase activity.

Specific activity measurements of the acyl-PCAPs were performed at 37 °C and contained 30 μM malonyl-PCAP, 30 μM acyl-PCAP (1 mm for the acyl-CoA series), and 5 μg of PfFabG in assay buffer (20 mM NaH₂PO₄, 300 mM NaCl, 80 μM NADPH, 1 mM DTT, pH 7.5) in a total volume of 100 μl. The reaction was started with the addition of 3 μg of PfFabBF. The course of the reaction was monitored for 1 min, and the rates of product formation were expressed as pmol/min. The substrate specificity of PfFabBF was additionally checked by CS-PAGE in order to exclude the possibility of PfFabG being the limiting factor.

Kinetic measurements were performed with C₄₀ to C₁₄₀-PCAPs under Michaelis-Menten conditions at 37 °C using various concentrations of acyl-PCAP (25, 50, 100, 200, 300, 400, 500, 600, and 800 μM), malonyl-PCAP at saturating conditions (400 μM), and 5 μg of PfFabG in assay buffer (final volume 100 μl). The kinetic parameters of malonyl-PCAP were determined using 500 μM C₄₀-PCAP and various concentrations of malonyl-PCAP (6.25, 12.5, 25, 50, 100, 200, and 400 μM). The reaction was started with the addition of 3 μg of PfFabBF. Control experiments (e.g. linearity of the rate of product formation at different PfFabBF concentrations) were performed to show that PfFabG is not the rate-limiting step in the reaction.

Cerulenin Inhibition Assay—Time dependence of cerulenin (CER) inhibition was analyzed by adding 3 μg of PfFabBF that had been pre-
incubated with 100 μM CER for 0, 10, 15, 20, 30, 45, 60, and 120 s to the assay buffer containing 30 μM malonyl-PfACP, 30 μM hexanoyl-PfACP, and 5 μg of PfFabG (total volume 100 μl). The IC50 value was determined using various concentrations of CER (0, 1, 10, 25, 50, and 100 μM) in the reaction mixture described above. The reaction was started without preincubation by the addition of 3 μg of enzyme. The irreversibility of CER inhibition was confirmed by adding inhibited enzyme to the reaction mixture described above, thereby diluting the CER concentration 20-fold. All measurements were performed at 37 °C.

Thiolactomycin Inhibition Assay—The IC50 value was determined as described for CER, except that the following thiolactomycin (TLM) concentrations were used: 0, 1, 10, 50, and 100 μM. The type of inhibition was determined with respect to malonyl-PfACP in consideration of the Michaelis-Menten steady state condition. To investigate the inhibition mechanism of TLM with respect to malonyl-PfACP, 250 μM C6:0-PfACP, various concentrations of malonyl-PfACP (10, 25, 50, 100, and 200 μM), and 1 μg of PfFabG were added to the assay buffer. The reaction was started by the addition of 3 μg of PfFabBF. The type of inhibition with respect to acyl-PfACP was determined as described for malonyl-PfACP, except that the C6:0-PfACP concentration was varied (50, 100, 200, 400, and 500 μM), and the malonyl-PfACP concentration was kept constant at 400 μM.

RESULTS

The fatty acid biosynthesis of P. falciparum received considerable attention with the recent discovery of this pathway in the parasite. In the meantime several key enzymes involved have been characterized with respect to their biochemical behavior as well as to their three-dimensional structure, and first inhibitors have been found or designed. However, the enzymatic characteristics of the β-ketoacyl-ACP synthase I/II...
homolog of the *P. falciparum* pathway have not been reported yet in the literature. The Plasmodium Genome Data base (32) proposed the open reading frame PFF1275c to be the mentioned β-ketoacyl-ACP synthase (PfFabBF). To confirm this hypothesis, we cloned the cDNA corresponding to PFF1275c and characterized its gene product PfFabBF.

**Multiple Sequence Alignment**—The open reading frame encodes a protein of 474 amino acids with an expected molecular mass of 52.6 kDa. PfFabBF has a long N-terminal extension that is characteristic of bipartite N-terminal presequences found in *Plasmodium* and *Toxoplasma* parasite proteins targeted to the apicoplast (5, 33). The size of the adjacent apicoplast translocation signal cannot be predicted and remains to be determined experimentally. A multiple sequence alignment of PfFabBF with FabB and FabF of *E. coli* and *A. thalana* is shown in Fig. 2. When compared with PfFabBF (residues 56–474), the parasite enzyme shares 31 and 38% identity with FabB (residues 1–406) and FabF (residues 1–412) of *E. coli* and 38 and 37% identity with FabB (residues 59–473) and FabF (residues 128–541) of *A. thalana*. The typical Cys/His/His motif found in β-ketoacyl-ACP synthases is also present in PfFabBF. This group entails the active site cysteine and histidines that are involved in the elongation reaction. In PfFabBF they correspond to Cys-221, His-362, and His-399 (Fig. 2).

**Expression and Purification of FabB**—Because of the lack of knowledge about the mature size of PfFabBF, the initial pET30b expression construct was designed to express a truncated enzyme missing the potential signal and translocation sequences, but retaining all amino acids needed to ensure complete functionality as deduced from sequence alignments with bacterial homologs. This initial pET30b vector containing the truncated pffabBF gene resulted in large quantities of protein that remained insoluble under any conditions. Subsequent refolding attempts achieved some solubilization, but the enzyme failed the activity tests, and it was shown by means of circular dichroism that it lacked secondary structure. At this stage a new clone (pET30b-PfFabBF) was prepared by site-directed mutagenesis to add back 6 amino acids derived from the N terminus that had been omitted in the first construct. Starting from this new construct, the extended insert was introduced into several pET vectors, aiming at the expression of the target protein with highly soluble fusion partners, i.e. glutathione S-transferase, thioredoxin, DsbA, and NusA. Only pET43.1b, which allows expression of the protein fused to the highly soluble NusA protein, resulted in a significant increase of soluble PfFabBF. The production of soluble fusion protein could be raised to acceptable amounts by subjecting the cells to osmotic stress (34, 35). We supplemented the TB medium with 1 mM betaine and sorbitol. Of all sorbitol concentrations tested (0, 330, and 660 mM and 1 M), 660 mM gave the best result. The identity of the fusion protein was confirmed by SDS-PAGE (Fig. 3A), Western blotting, and thrombin digestion. The first purification step of the fusion protein consisted of loading the clarified soluble fraction on a HisTrap chelating column. NusA-fusion proteins from pET43 are known to bind rather weakly to the nickel column, making a complete elimination of contaminating proteins difficult. PfFabBF eluted at an imidazole concentration of 125 mM and was of low purity, as expected. Most contaminants were eliminated by cation exchange chromatography, resulting in a preparation of about 80% purity as deduced from SDS-PAGE and gel densitometry. A final polishing step using gel filtration chromatography could increase purity to >95% but resulted in >50% loss of protein. Various control experiments showed clearly that the impurities present in the sample do not interfere with the measurement (i.e. do not introduce unspecific oxidase activity). In addition, the NusA-PfFabBF fusion protein displayed identical activities compared with the thrombin-cleaved PfFabBF. Based on these results, we used the more stable fusion protein for all measurements. The purification typically resulted in 2–3 mg of PfFabBF fusion protein/liter of media.

**Purification of PfACP and Preparation of Acylated Substrates**—Expression of PfACP in BL21(DE3)-CodonPlus-RIL cells and subsequent purification by nickel affinity chromatography yielded 20–30 mg of highly pure protein/liter culture. Thrombin-digested apo- and holopfACP were separated by anion exchange chromatography (Fig. 3B). Pure apo-pfACP was used for acylation by EeACPs. Under the experimental conditions described, the acylation of PfACP is complete within 2 min when acyl-CoAs with chain lengths up to C14:0 were used. The reaction was considerably slower with C16:0-CoA; thus the incubation time was extended to 1 h to ensure complete acylation. Purity of the acyl-CoAPs was verified by matrix-assisted laser desorption ionization time-of-flight-mass spectrometry and by CS-PAGE (Fig. 3C).

**PfFabBF Activity with Acyl-pfACP and Acyl-CoA as Substrates**—To examine the substrate acceptance, we tested C4:0- to C16:0-acyl-pfACP and the corresponding acyl-CoAs. PfFabBF readily elongates C4:0 through C10:0-pfACPcs, has significantly less activity with C12:0- and C14:0-pfACPcs, and no capacity for elongation of C16:0-pfACP (Table 1). The acyl-CoAs are much poorer substrates compared with acyl-pfACPcs. When measured at 30 μM, the activity of PfFabBF with C4:0- to C14:0-CoAs as substrates was in the range of the background, but a trend toward higher activity with C16:0- and C18:0-CoAs could be observed. At acyl-CoA concentrations of 1 mM we detected activity with C4:0- to C14:0-CoAs. PfFabBF was found to be more than twice as active with C16:0- and C18:0-CoAs compared with the shorter chain acyl-CoAs (Table 1). Thus PfFabBF accepts long chain acyl-CoAs but not long chain acyl-pfACPcs, and the long chain acyl-CoAs seem to be better substrates than the shorter chain acyl-CoAs.

**Kinetic Parameters of PfFabBF Substrates**—Kinetic parameters were determined for C4:0- to C16:0- and malonyl-pfACP (Fig. 4A). The *Km*
The values obtained for C12:0 and C14:0 are considerably lower, exhibiting decreased catalytic efficiencies by 40–50% (Fig. 4). The results corroborate the findings obtained at the PfFabBF activity test with different substrates.

**TABLE 2**

| Substrate          | $K_m$ | $V_{max}$ | $k_{cat}$ | $k_{cat}/K_m$ |
|--------------------|-------|-----------|-----------|---------------|
| Substrate          | $\mu M$ | $\mu M/min$ | $\mu M/min$ | $\mu M/min$ |
| C4:0-PfACP (CoA)   | 211.1 | 2.2 ± 0.06 | 79.1 ± 2.0 | 0.37 ± 0.01 |
| C6:0-PfACP (CoA)   | 115.6 | 1.2 ± 0.03 | 57.6 ± 1.5 | 0.50 ± 0.02 |
| C8:0-PfACP (CoA)   | 198.2 | 2.7 ± 0.05 | 97.8 ± 1.9 | 0.49 ± 0.02 |
| C10:0-PfACP (CoA)  | 403.4 | 5.2 ± 0.40 | 184.0 ± 1.3 | 0.46 ± 0.03 |
| C12:0-PfACP (CoA)  | 196.4 | 1.2 ± 0.10 | 41.1 ± 0.6 | 0.21 ± 0.01 |
| C14:0-PfACP (CoA)  | 437.0 | 2.2 ± 0.20 | 76.7 ± 0.7 | 0.18 ± 0.02 |
| C16:0-PfACP (CoA)  | NDA   | NDA       | NDA       | NDA           |

* NDA indicates no detectable activity.

**DISCUSSION**

In contrast to most other eukaryotes, Plasmodia harbor a type II fatty acid biosynthesis pathway. Because of structural differences to the eukaryotic system, the enzymes of FAS-II represent interesting new targets for the development of novel antimalarials. The unique elongation condensing enzyme in *P. falciparum*, PfFabBF, is representative of these promising targets.

Multiple sequence alignments of PfFabBF with $\beta$-ketoadyl-ACP synthases of other organisms display the homology of the enzymes, with 30–40% identical amino acids. As PfFabBF is the unique elongating enzyme remained inactive. Because CER binds covalently to PfFabBF, leading to a complete inactivation of the enzyme within 1 min (Fig. 5A). The irreversibility of CER inhibition was confirmed by diluting the CER concentration 20-fold. In case of a reversible inhibitor, the enzyme would have regained activity. This was not the case with CER as the enzyme remained inactive. Because CER binds covalently to PfFabBF, Fig. 5B reflects the rate of complex formation at different inhibitor concentrations. The IC$_{50}$ value was determined to be 15.8 ± 2.3 µM under the conditions applied.

TLM inhibits PfFabBF reversibly with an IC$_{50}$ of 23.6 ± 6.0 µM (Fig. 6A). TLM is a competitive inhibitor with respect to malonyl-PfACP (Fig. 6B). Preliminary experiments indicate uncompetitive inhibition with respect to acyl-PfACP (data not shown). Analysis of the data according to Dixon and secondary plots resulted in a $K_i$ value of 10 ± 4 µM.

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**TABLE 1**

PfFabBF activity with acyl-PfACP and acyl-CoA substrates

| Substrate          | Activity $^a$ | Activity $^b$ | Activity $^c$ |
|--------------------|---------------|---------------|---------------|
| C4:0-PfACP (CoA)   | 94.8 ± 6.6    | (36.0 ± 10.9) |               |
| C6:0-PfACP (CoA)   | 104.9 ± 9.1   | (23.7 ± 4.3)  |               |
| C8:0-PfACP (CoA)   | 66.8 ± 1.4    | (36.8 ± 3)    |               |
| C10:0-PfACP (CoA)  | 97.5 ± 9.7    | (36.6 ± 7.6)  |               |
| C12:0-PfACP (CoA)  | 30.7 ± 4.2    | (15.2 ± 9.2)  |               |
| C14:0-PfACP (CoA)  | 24.0 ± 0.6    | (26.7 ± 17.3) |               |
| C16:0-PfACP (CoA)  | NDA $^d$      | (100.0 ± 20.2)|               |
| C18:0-PfACP (CoA)  | NDA $^d$      | (88.8 ± 14.6) |               |

* Acyl-PfACP substrates are measured at 30 µM, and 1 µM of the corresponding CoA derivatives (shown in parentheses) is applied.
* The activity towards the corresponding CoA substrates is shown in parentheses.
* The results are the mean of duplicate experiments.

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**FIGURE 4.** Kinetic analysis of PfFabBF with acyl-PfACPs. A, the initial velocities of product formation were determined with increasing concentrations of acyl-PfACP. The data were analyzed by nonlinear regression analysis (Michaelis-Menten model) using Origin software. The average of two data points has been plotted. C, C4-0-PfACP; C, C6-0-PfACP; C, C8-0-PfACP; C, C10-0-PfACP; C, C12-0-PfACP; C, C14-0-PfACP; C, C16-0-PfACP; C, the catalytic efficiency of PfFabBF for C4-0 to C16-0-PfACP was calculated from $k_{cat}$ and $K_m$ values. PfFabBF exhibits maximum activity with C4-0 to C12-0-PfACP as substrates. The results reflect the mean of duplicate experiments.

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**FIGURE 5.** Kinetic analysis of PfFabBF with acyl-PfACPs. B, the catalytic efficiency of PfFabBF for C4-0 to C16-0-PfACP was calculated from $k_{cat}$ and $K_m$ values. PfFabBF exhibits maximum activity with C4-0 to C12-0-PfACP as substrates. The results reflect the mean of duplicate experiments.

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**TABLE 2**

Kinetic parameters of acyl-PfACPs

### Conclusion

Described above, despite the limited informational content of measurements performed at single substrate concentrations.

Effects of CER and TLM on PfFabBF—CER is known to be an irreversible inhibitor of $\beta$-ketoadyl-ACP syntheses. To investigate the sensitivity of PfFabBF to CER, the enzyme was preincubated with 100 µM CER. Samples were taken at various time points and assayed for activity. As expected, CER was found to be a potent inhibitor of PfFabBF activity, leading to a complete inactivation of the enzyme within 1 min (Fig. 5A). The irreversibility of CER inhibition was confirmed by diluting the CER concentration 20-fold. In case of a reversible inhibitor, the enzyme would have regained activity. This was not the case with CER as the enzyme remained inactive. Because CER binds covalently to PfFabBF, Fig. 5B reflects the rate of complex formation at different inhibitor concentrations. The IC$_{50}$ value was determined to be 15.8 ± 2.3 µM under the conditions applied.

TLM inhibits PfFabBF reversibly with an IC$_{50}$ of 23.6 ± 6.0 µM (Fig. 6A). TLM is a competitive inhibitor with respect to malonyl-PfACP (Fig. 6B). Preliminary experiments indicate uncompetitive inhibition with respect to acyl-PfACP (data not shown). Analysis of the data according to Dixon and secondary plots resulted in a $K_i$ value of 10 ± 4 µM.
that the PfFabBF mechanism of acyl-enzyme formation, decarboxylation, and condensation is very similar to that of other organisms and that β-ketoacyl-ACP synthase inhibitors CER and TLM exhibit identical binding modes. Structural and biochemical analyses of PfFabBF and its interaction with CER and TLM will be helpful in providing clues for the development of new compounds that selectively target PfFabBF. We therefore established an expression system that allowed production of soluble and active PfFabBF. Production of soluble protein was only achieved by expressing the NusA-PfFabBF fusion protein under increased osmotic pressure (34, 35). It is assumed that subjecting the cells to osmotic stress by addition of sorbitol to the growth medium facilitates the uptake of the "compatible osmolyte" betaine. Betaine is believed to stabilize protein structure by minimizing solvent-protein contacts (35, 38).

PfFabBF was assayed for activity with acyl-PfACP substrates as well as with the acyl-CoA derivatives to investigate chain length specificity of PfFabBF for natural and artificial substrates. Acyl-PfACPs with chain lengths ranging from C4:0 to C16:0 were analyzed. PfFabBF readily elongates C6:0 to C10:0-PfACP. The enzyme is significantly less active with C4:0-PfACP, and the catalytic efficiency decreases even more with the use of C12:0 and C14:0-PfACP. Elongation of C16:0 was not detected at all. The observed distribution of the catalytic efficiency indicates maximum PfFabBF activity for C6:0-, C8:0-, and C10:0-PfACP substrates.

Investigation of the kinetic parameters of the acyl-PfACP substrates revealed that the decreases in catalytic efficiency with C12:0 and C14:0-PfACPs are caused by a slower conversion of the [ES] complex to free enzyme and product (E + P) in the case of C12:0-PfACP and by a decreased affinity of the substrate for PfFabBF in the case of C14:0-PfACP. C12:0 has a Km of 196 μM, which is in the range of the Km values found for shorter chain acyl-PfACP. Here the lower catalytic efficiency is because of a lower Vmax value. For C14:0-PfACP, an increase in Km to 437 μM is observed, indicating a decreased affinity for the enzyme. C10:0-PfACP shows an interesting behavior; it is an efficient substrate despite a Km of 403 μM, because of a high Vmax value. These data are consistent with the recent analysis of parasite fatty acid synthesis using cell-free extracts. It was found that mainly C12 to C14 fatty acids were synthesized in P. falciparum (39).

We also tested the specific activities of PfFabBF with acyl-CoAs. They are poor substrates with C4:0- to C14:0-CoAs being 100–150 times less active than the corresponding acyl-PfACPs. This is in agreement with the general observation that β-ketoacyl-ACP synthases exhibit low specific activity with acyl-CoA derivatives (16, 40). Surprisingly, among all acyl-CoAs tested, PfFabBF displayed the highest activity for C16:0-CoA and C18:0-CoA. This is in direct contrast to the results obtained for the natural acyl-PfACP substrates that exhibit no activity with C16:0 and C18:0 acyl chain length. Investigation of the substrate acceptance of other β-ketoacyl-ACP synthases with a series of acyl-ACPs and the corresponding acyl-CoA derivatives is not described in literature, and thus it is...
not known whether this behavior is also common for other \(\beta\)-ketoacyl-ACP-synthases. However, as the activity of acyl-CoAs is very low and high substrate concentrations had to be applied, PfFabBF activity with respect to acyl-CoA is not likely to be physiologically relevant.

Given the very different substrate specificity of PfFabBF toward its natural and artificial substrates, it is likely that acyl-PfACP and acyl-CoA display different behavior also with the other enzymes involved in fatty acid biosynthesis. Thus, to avoid artifacts, acyl-PfACP should be used for characterizing enzymes in the \(P. falciparum\) type-II FAS system. This most probably also applies to FAS systems of other organisms.

Comparing the kinetic parameters of PfFabBF to the kinetic parameters of corresponding enzymes of other organisms is difficult due to the high substrate concentrations that had to be applied. PfFabBF activity with respect to PfACP was about 100-fold greater than the one reported for other enzymes of type-II fatty acid biosynthesis. Thus, to avoid artifacts, acyl-PfACP should be used for characterizing enzymes in the \(P. falciparum\) type-II FAS system. This most probably also applies to FAS systems of other organisms.

The presented biochemical characterization of PfFabBF provides insight into the catalytic mechanism of PfFabBF. With the development of a system to produce soluble and active PfFabBF, it is now possible to extend the screening for inhibitors on the \(\beta\)-ketoacyl-ACP synthase activity of \(P. falciparum\). Further investigation of the biochemical properties and structure of PfFabBF could form the basis for the rational design of new lead compounds.

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