Identification, Characterization, and Inhibition of *Plasmodium falciparum* β-Hydroxyacyl-Acyl Carrier Protein Dehydratase (FabZ)*

Shailendra Kumar Sharma‡§, Mili Kapoor‡§, T. N. C. Ramya‡, Sanjay Kumar‡, Gyanendra Kumar‡, Rahul Modak‡, Shilpi Sharma‡, Namita Suroliya‡, and Avadhesh Surolia‡**

From the ‡Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India and the ¶Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, India

The emergence of drug-resistant forms of *Plasmodium falciparum* emphasizes the need to develop new antimarialars. In this context, the fatty acid biosynthesis (FAS) pathway of the malarial parasite has recently received a lot of attention. Due to differences in the fatty acid biosynthesis systems of *Plasmodium* and man, this pathway is a good target for the development of new and selective therapeutic drugs directed against malaria. In continuation of these efforts we report cloning and overexpression of *P. falciparum* β-hydroxyacyl-acyl carrier protein (ACP) dehydratase (PfFabZ) gene that codes for a 17-kDa protein. The enzyme catalyzes the dehydration of β-hydroxyacyl-ACP to trans-2-acyl-ACP, the third step in the elongation phase of the FAS cycle. It has a *Km* of 199 μM and *kcat/Km* of 80.4 M⁻¹ s⁻¹ for the substrate analog β-hydroxybutyryl-CoA but utilizes crotonoyl-CoA, the product of the reaction, more efficiently (Km = 86 μM, kcat/Km = 220 M⁻¹ s⁻¹). More importantly, we also identify inhibitors (NAS-91 and NAS-21) for the enzyme. Both the inhibitors prevented the binding of crotonoyl-CoA to PfFabZ in a competitive fashion. Indeed these inhibitors compromised the growth of *P. falciparum* in cultures and inhibited the parasite fatty acid synthesis pathway both in cell-free extracts as well as in situ. We modeled the structure of PfFabZ using Escherichia coli β-hydroxydecanoyl thioester dehydratase (EcFabA) as a template. We also modeled the inhibitor complexes of PfFabZ to elucidate the mode of binding of these compounds to FabZ. The discovery of the inhibitors of FabZ, reported for the first time against any member of this family of enzymes, essential to the type II FAS pathway opens up new avenues for treating a number of infectious diseases including malaria.

Malaria continues to exact the highest mortality and morbidity rate next only to tuberculosis. “The scourge of the tropics,” malaria is endemic to around 100 countries in the world.

* This work was supported by a grant from the Department of Biotechnology, Government of India (to N.S.) and in part by Shantha Biotechnics Pvt. Ltd., Hyderabad, India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence may be addressed. Tel.: 91-80-2932389; Fax: 91-80-2932389; E-mail: surolia@jncasr.ac.in.

† Both authors made equal contributions to this work.

‡ The abbreviations used are: FAS, fatty acid synthesis; FabZ, β-hydroxyacyl-ACP dehydratase; ACP, acyl carrier protein; PfFabZ, *Plasmodium falciparum* β-hydroxyacyl-ACP dehydratase; PIFabZ, *Plasmodium* enoyl-ACP reductase; FabA, β-hydroxydecanoyl thioester dehydratase; RT, reverse transcriptase; Ni-NTA, nickel-nitrilotriacetic acid; HPLC, high pressure liquid chromatography; EcFabA, *Escherichia coli* β-hydroxydecanoyl thioester dehydratase.

Approximately 500 million cases of malaria are reported every year, and around 3000 children die of malaria every day (1). Emerging resistance to chloroquine and other currently prescribed drugs limits treatment of malaria today, in particular cerebral malaria, caused by *Plasmodium falciparum* (2, 3). The situation definitely warrants express remedial actions: extensive research on *P. falciparum* to identify drug targets and, ultimately, the development of a new armamentarium of antimalarials.

Our recent demonstration of the occurrence of the type II fatty acid synthesis (FAS) pathway in the malaria parasite and its inhibition by triclosan, an inhibitor of the rate-limiting enzyme of type II FAS, enoyl-acyl carrier protein (ACP) reductase, proved the pivotal role played by this pathway in the survival of the malarial parasite (4, 5). The essential role of fatty acids and lipids in cell growth and differentiation and the different type (type I) of fatty acid biosynthetic pathway occurring in the human host, which is distinct from type II FAS of the malaria parasite, makes this pathway an attractive drug target for treating malaria (6, 7).

The type II fatty acid biosynthesis pathway, found in most bacteria and plants, is typified by the existence of distinct enzymes encoded by unique genes for catalyzing each of the four individual chemical reactions required to complete successive cycles of fatty acid elongation (Refs. 4, 8, and 9 and Scheme I). This is in contrast to the type I FAS characterized by a multifunctional enzyme catalyzing all the steps of the pathway (10). The third step of the elongation cycle, the dehydration of β-hydroxyacyl-ACP to trans-2-acyl-ACP, is carried out by FabA or FabZ (11). While FabZ only catalyzes the dehydration reaction, FabA is a bifunctional enzyme and carries out an additional isomerization reaction of trans-2-acyl-ACP to cis-3-acyl-ACP, a reaction essential to unsaturated fatty acid synthesis (12). In *Streptococcus pneumoniae*, instead of FabA, a new enzyme, FabM (trans-2,cis-3-decenoyl-ACP isomerase), catalyzes the isomerization step of trans-2-decenoyl-ACP to cis-3-decenoyl-ACP following the dehydration step by FabZ (13). FabZ is the primary dehydratase that participates in the elongation cycles of saturated as well as unsaturated fatty acid biosynthesis, whereas FabA is more active in the dehydration of β-hydroxydecanoyl-ACP (11, 14). This dichotomy allows the...
The condensation of acetyl-CoA and malonyl-ACP in the first reaction of the elongation phase of fatty acid synthesis is catalyzed by β-ketoacyl-ACP synthase III (FabH). The reduction of the resulting β-ketoester by β-ketoacyl-ACP reductase (FabB) forms β-hydroxyacyl-ACP, which serves as a substrate for FabZ. The dehydration by FabZ generates a,β-unsaturated acyl-ACP that is a substrate of enoyl-ACP reductase ( Fab), the product of which reenters the cycle by condensation with malonyl-ACP catalyzed by β-ketoacyl-ACP synthase III (FabB). For the synthesis of unsaturated fatty acids, the dehydration and subsequent isomerization of β-hydroxyacyl-ACP by β-hydroxydecanoyl thioester dehydratase (FabA) generates cis-3 unsaturated product, which can again enter the cycle upon condensing with malonyl-ACP by the action of FabB. ACP, acyl carrier protein; CoA, coenzyme A.

synthesis of unsaturated as well as saturated fatty acids, which eventually dictate the composition of the cell membranes. Dehydratase activity is crucial for the supply of trans-2-acyl-ACP to FabI, which pulls each cycle of elongation to completion (11, 15). FabZ thus presents itself as a suitable, yet unexplored target for the design of antimalarials.

Here we report the cloning, expression, and characterization of \textit{P. falciparum} FabZ (PfFabZ), the gene sequence of which has been deposited earlier by us in a public data base (GenBank™ accession number AY118082). FabZ was expressed as a soluble, active protein, and its purification was achieved by a single step purification protocol. We report its molecular and enzymatic properties. Further we have identified two lead compounds with inhibitory activity toward \textit{P. falciparum} FabZ, which represents a significant advance in this area as no inhibitors of FabZ from any source are known to date.

**EXPERIMENTAL PROCEDURES**

**Materials**

Crotosylo-CoA, \(\beta\)-hydroxybutyryl-CoA, imidazole, kanamycin, and SDS-PAGE reagents were obtained from Sigma. Cesium carbonate, 2-bromo-4-chlorophenol, \(N\)-methylpyrrolidinone, and cuprous chloride were purchased from Aldrich. Media components were obtained from Hi-media (Delhi, India). All other chemicals used were of analytical grade. \(1,2\,-\text{C}^{14}\) Acetic acid, sodium salt (specific activity, 60 mcCi/mmol), and \(2\,-\text{C}^{14}\) Malonyl-CoA (specific activity, 54.2 mcCi/mmol) were obtained from PerkinElmer Life Sciences.

**Strains and Plasmids**

\textit{Escherichia coli} DH5α cells were used during the cloning of the gene. pET-28a(+) vector (Novagen) and BL21(DE3) cells (Novagen) were used for the expression of FabZ.

**Cloning of PfFabZ in \textit{E. coli}**

Total RNA was isolated from 10 ml of packed erythrocytes (infected with \textit{P. falciparum}, 10–12% parasitemia) after saponin lysis by a single step method of RNA isolation (16). The isolated RNA was treated with RNase-free DNase (Promega, 1 unit/μg of RNA) for 45 min at 37 °C and repurified by phenol-chloroform extraction and ethanol precipitation. RT-PCR was performed using a one step RT-PCR kit (Qiagen, Valencia, CA). PCR was performed with the primers (forward, 5′-GGATATCATATGAAATTCGCTTCCATATTGAT-3′, and reverse, 5′-CCGGATCTTATATCGATATGGCAAGCTGTCATTCC-3′ with NdeI and BamHI sites underlined, respectively). PCR conditions used were: 1 × (94 °C 5 min), 30 × (94 °C 1 min, 50 °C 1 min, 72 °C 1 min), 1 × (72 °C 10 min). The primers were designed using the GenBank™ accession number AF237572 to clone the mature protein (without the leader peptide and transit sequence, required for targeting of the protein to apicoplast). The 465-bp RT-PCR product was excised from 1.2% agarose gel, purified using silica gel particles (QIAEX II gel extraction system, Qiagen), and cloned in pGEMT-Easy vector (Promega). Candidate plasmids containing the correct sized inserts were confirmed by digestion and dyeo sequencing on an ABI Prism 377 semiautomated sequencer Version 3.0.

**Expression of PfFabZ**

The insert present in pGEMT-Easy was reamplified using the above-mentioned primers and subcloned in pET-28a(+) vector (Novagen) in-frame with the N terminus His tag. The constructs were transformed into \textit{E. coli} BL21(DE3) cells (Novagen), and cultures were grown at 37 °C in Luria Broth (Hi-media) until A_{600} of 0.6. These were induced with 1 mM isopropyl-\(\beta\)-thiogalactopyranoside and further incubated at 12 °C for 12 h. Cells were harvested at 8000 rpm for 10 min, and the resultant pellet was stored at −70 °C until further use.

**Purification of PfFabZ**

The pellet was resuspended in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 0.5 M NaCl, and 5 mM imidazole. Cells were disrupted using a probe-type ultrasonicator (Vibra-Cell, Sonics and Materials). Cell debris were removed by centrifugation at 15,000 rpm for 30 min. The supernatant obtained was applied to a Ni-NTA metal affinity column (His-bind resin, Novagen) equilibrated with the lysis buffer. The column was initially washed with lysis buffer and subsequently washed with the same buffer containing 60 mM imidazole. The protein was eluted using a step gradient of 0.3–0.5 M imidazole, and fractions were tested for purity by SDS-PAGE. The protein was applied on a fast protein liquid chromatography desalting column to remove imidazole followed by concentration of the protein using Centrifrip-10. Protein concentration was determined from A_{280}, assuming the molar extinction coefficient E_{280} = 9530 M^−1 cm^−1, which was calculated using the formula from Ref. 17.

We further truncated the protein by removing 10 residues from the N terminus by using primers (forward, 5′-AGCGTACATGGTCATCAGTCATCATCATTATATTGATAGATAGATAGATTAGAAATTCTTTCCACATGATACTCCCTCCCTCC-3′, and reverse, 5′-CGGGATCTTATATCCGATCTTCCGATCTTCC-3′ with NcoI and BamHI sites underlined, respectively), and the PCR conditions used were as described above. The truncated gene was ligated into pET-28a(+) vector. The truncation led to an increase in the protein yield by 3-fold, i.e. from 5 to 15 mg/liter.

**Dynamic Light Scattering Studies of PfFabZ**

Purified PfFabZ (2 mg/ml) was injected onto a Superdex™ 200 HR 10-×300-mm column (Amersham Biosciences) equilibrated in 20 mM Tris, 500 mM NaCl (pH 7.5) connected to an AKTA™ design system. The column flow rate was maintained at 0.5 ml/min. The molecular weight of PfFabZ was determined by plotting V/Vc versus elution volume for standard proteins. Vc corresponds to the peak elution volume of the standard protein, and V represents the void volume of the column determined using blue dextran (M<sub>0</sub> < 2,000,000).

Dynamic light scattering studies were performed on a Brookhaven Instruments Dynamic Light Scattering setup that can measure sizes from 2 to 4000 nm. The sample of PfFabZ (1 mg/ml) in 20 mM Tris, 500 mM NaCl (pH 7.5) was centrifuged at 14,000 rpm for 15 min and filtered through a 0.2-μ filter. The data acquisition time was 3 min. The routines used to fit the data points were cumulants, and non-negative least squares analysis was used to obtain the radius of gyration of PfFabZ.

**Spectrophotometric Assay for Determining Enzyme Activity**

All experiments were carried out on a Jasco V-530 UV-visible spectrophotometer. FabZ was assayed at 25 °C by monitoring the forward as well as the reverse reaction, i.e. increase and decrease in A_{280}, respectively, due to the conversion of \(\beta\)-hydroxybutyryl-CoA to crotosylo-CoA and vice versa. The standard reaction mixture in a total volume of 100
The reaction was performed as described above and stopped by the addition of 3% chloroform, and the compounds were separated by reverse phase HPLC as described earlier (18). Briefly, the mixtures were loaded onto a Sephasil Peptide C18 column (4.6 mm x 25 cm; particle diameter, 5 μm; column volume, 4.155 ml; Amersham Biosciences), and the compounds were separated on a gradient of 220 mM phosphate buffer, pH 4.0 and methanol:chloroform (98:2). The retention times of -hydroxybutyryl-CoA, crotonoyl-CoA, butyryl-CoA, and NADH were 14.8, 39.61, 48.47, and 4.31 min, respectively, at a flow rate of 0.5 ml/min. To determine the K_m of PfFabZ for crotonoyl-CoA by HPLC, the peaks obtained while running different reaction mixtures were compared with the peaks of the standard run.

**HPLC Assay**

The reaction was performed as described above and stopped by the addition of 3% chloroform, and the compounds were separated by reverse phase HPLC as described earlier (18). Briefly, the mixtures were loaded onto a Sephasil Peptide C18 column (4.6 mm x 25 cm; particle diameter, 5 μm; column volume, 4.155 ml; Amersham Biosciences), and the compounds were separated on a gradient of 220 mM phosphate buffer, pH 4.0 and methanol:chloroform (98:2). The retention times of β-hydroxybutyryl-CoA, crotonoyl-CoA, butyryl-CoA, and NADH were 14.8, 39.61, 48.47, and 4.31 min, respectively, at a flow rate of 0.5 ml/min. To determine the K_m of PfFabZ for crotonoyl-CoA by HPLC, the peaks obtained while running different reaction mixtures were compared with the peaks of the standard run. For the
FabZ-FabI coupled assay, PfFabI was purified according to published protocols (5).

Modeling of P. falciparum FabZ

The structure of FabZ is not available from any source, but P. falciparum FabZ shares 21% sequence identity with E. coli FabA. We modeled P. falciparum FabZ using E. coli FabA (Protein Data Bank code 1MKB) as the template. Modeling was done using MOE (Molecular Operating Environment) (19). Ten intermediate homology models were built as a result of the permutational selection of different loop candidates and side chain rotamers. The intermediate models were averaged to produce the final model by Cartesian average. We used Swiss-Pdb-Viewer to generate the dimeric structure with transformation matrix from the template structure (20).

Synthesis of Inhibitors

Of the four compounds synthesized, NAS-21, NAS-75, and NAS-79 were synthesized using published procedures (21, 22). NAS-91 is a novel compound, therefore, its synthesis is described in detail below.

Preparation of 4,4,4-Trifluoro-1-(4-nitrophenyl)butane-1,3-dione (NAS-21)—To a solution of ethyl trifluoroacetate (1.4 ml, 0.012 M) in methyl tert-butyl ether 25% NaOCH₃ in methanol (4 ml, 1.5 eq) was added slowly. A solution of 4-nitroacetophenone (1.65 g, 0.01M) in methyl tert-butyl ether was added to this mixture dropwise over 10 min. The reaction mixture was stirred at room temperature for 18 h. After the completion of the reaction the mixture was quenched with 3N HCl. The organic layer was collected, washed with water and brine, and dried over Na₂SO₄, and the product (1.80 g) was crystallized from chloroform (70% yield).

Preparation of 1-(4-Methoxyphenyl)ethanone [(4-Trifluoromethyl)pyrimidine-2-yl]hydrazone (NAS-75)—To 4-methoxyacetophenone (150 mg, 1 mM) in 5 ml of acetic acid and 1 ml of water 2-hydrazino-4-(trifluoromethyl)pyrimidine (178 mg, 1 mM) was added. Shortly after addition, a solid formed to which acetic acid was added to maintain stirring for 16 h. The reaction mixture was diluted with 4 ml of water, and subsequently the solid mass was filtered and dried in vacuo to afford 270 mg (90% yield) of the product.

Preparation of 1-(4-Methylphenyl)ethanone [(4-Trifluoromethyl)pyrimidine-2-yl]hydrazone (NAS-79)—To 4-methoxyacetophenone (150 mg, 1 mM) in 5 ml of acetic acid and 1 ml of water 2-hydrazino-4-(trifluoromethyl)pyrimidine (178 mg, 1 mM) was added. Shortly after addition, a solid formed to which acetic acid was added to maintain stirring for 16 h. The reaction mixture was diluted with 4 ml of water, and subsequently the solid mass was filtered and dried in vacuo to afford 270 mg (90% yield) of the product.

Preparation of 4-Chloro-2-[(5-chloroquinolin-8-yl)oxy]phenol (NAS-91)—Cesium carbonate (2 mM, 651 mg) was added to 5-chloro-8-hy-
The completion of the reaction was monitored by spectrophotometric assay performed as described above except that a given inhibitor was added prior to the initiation of the reaction by addition of crotonoyl-CoA. The studies were performed in the presence of 1% MeSO used for solubilizing the inhibitors. The inhibition constant $K_i$ was determined by the Dixon plot and the Lineweaver-Burk equation. In Dixon’s method (23), the enzyme activity was measured at four different concentrations of substrate (50, 75, 100, and 125 mM) as a function of the inhibitor concentration (0.1–2.5 $\mu$M). For the determination of $K_i$ by the Lineweaver-Burk equation the enzyme assays were performed in the absence and presence (1 $\mu$M) of each inhibitor at a range of substrate concentrations (10–60 $\mu$M).

**Fluorescence Analysis**

Fluorescence measurements were performed on a Jobin-Yvon Horiba fluorimeter under computer control. The excitation and emission monochromator slit widths were 3 and 5 nm, respectively. Measurements were performed at 25 $^\circ$C in a 3-m1 quartz cuvette, and the solutions were mixed continuously on a magnetic stirrer. The solutions containing PfFabZ were excited at 280 nm, and the emission was recorded from 300 to 500 nm.

For inhibitor binding studies, PfFabZ (22 $\mu$M) in 20 mM Tris, 500 mM NaCl, pH 7.5 was titrated with different concentrations of the inhibitors (0–8.3 $\mu$M). The magnitude of fluorescence decrease ($F_0 - F$) upon addition of each inhibitor concentration was fitted to Equation 2 to determine the value of $K_i$.

$$F_0 - F = \Delta F_{\text{em}}/I + (K_i/I)$$  

Equation 2

Corrections for the inner filter effect were performed according to Equation 3 (24).

$$F = F_0 \text{antilog}[(A_{\text{em}} + A_{\text{ex}})/2]$$  

Equation 3

In the absence and presence (1 $\mu$M) of each inhibitor at a range of substrate concentrations (10–60 $\mu$M).

**Inhibition of PfFabZ Activity**

The inhibition of PfFabZ activity was monitored by the spectrophotometric assay described above except that a given inhibitor was added prior to the initiation of the reaction by addition of crotonoyl-CoA. The studies were performed in the presence of 1% crotonoyl-CoA, $\beta$-hydroxybutyryl-CoA, and $\beta$-hydroxybutyryl-CoA. The studies were performed in the presence of 1% diphenylamine (1 mM, 207 mg) and copper (I) chloride (0.5 mM, 50 mg) was added. The flask was degassed and filled with N2 four to five times. The reaction mixture was heated at 140 $^\circ$C. The reaction mixture was heated at 140 $^\circ$C. The reaction mixture was heated at 140 $^\circ$C.

**Kinetic Analysis of PfFabZ with crotonoyl-CoA**

The initial velocities of product formation were determined with increasing concentrations of crotonoyl-CoA. The studies were performed in the presence of 1% crotonoyl-CoA and $\beta$-hydroxybutyryl-CoA, respectively. Similar values were obtained upon analysis of the data by Lineweaver-Burk plots ($K_m$) and $K_i$.

**Growth Inhibition Assay**

The experiments were performed using $P. falciparum$ FCK2 strain (chloroquine-sensitive, $IC_{50}$ 18 nm), an isolate from Karnataka, India. $P. falciparum$ was cultured using standard techniques (25) and routinely synchronized using 5% sorbitol (26). Growth inhibition by the compounds of interest was assessed by the $\Delta$GPyHxanthine uptake. Typically uninfected or infected (1–2% parasitemia) red blood cells (2% hematocrit) were added to the culture medium in the wells of a 96-well plate (Nunc), and different concentrations of inhibitor in MeSO were added such that the final concentration of MeSO did not exceed 0.05%.

For the determination of the value of $K_i$, the change in free energy upon binding of inhibitor to the protein, was calculated from Equation 4.

$$\Delta G = -RT\ln K_i$$  

Equation 4

where $R$ is the gas constant, $T$ is the temperature in Kelvin, and $K_i$ is the binding constant.

**Cell-free Fatty Acid Synthesis by $P. falciparum$ Extracts**

The in vitro fatty acid synthesis was performed as described earlier (4, 30). Trophozoites isolated from 100-m1 cultures with 8–10% parasitemia were resuspended and sonicated in 0.2 ml of 70 mM potassium phosphate buffer (pH 7.0) for 5 s and centrifuged at 48,000 $\times$ g for 1 h.

The supernatant fraction was used as crude extract for determining the in vitro fatty acid synthesis. The assay mixture in 70 mM potassium phosphate (pH 7.0) contained 1.4 mM dithiothreitol, 20 mM acetyl-CoA, 3.6 mM glucose 6-phosphate, 0.14 mM EDTA, 200 µM NADH, 200 µM NADPH, 1 unit of glucose-6-phosphate dehydrogenase, 250 µg of parasite protein, and 80 µM [2-14C]malonyl-CoA (4). The reaction was initiated by addition of solution containing 120 µg of freshly reduced ACP. The reaction mixture was incubated for 35 min with or without 10 µM NAS-91 or NAS-21, which was added just before the addition of ACP, at 37°C. The reaction mixture was then treated with 4 M HCl at 100°C for 2 h. The fatty acids were extracted in chloroform and methylated at 4°C with diazomethane in ether, and the incorporation of [2-14C]malonyl-CoA into fatty acids was monitored by thin layer chromatography on silanized silica thin layer plates (Merck) developed with a solvent system of acetone:water:methanol:acetic acid (70:50:35:1) as described previously (4, 30).

Incorporation of [1,2-14C]Acetic Acid into Fatty Acids in P. falciparum Cultures

Inhibitors (10 and 100 µM) were added to P. falciparum cultures (100 ml, 9–10% parasitemia) for 70 h after which cultures were resuspended in 7 ml of the complete medium while retaining the same concentrations of the inhibitors. To this [1,2-14C]acetic acid was added (50 µCi/ml) (4). After 2 h, parasites were isolated, washed thoroughly with phosphate-buffered saline, lysed, sonicated, spotted onto a Whatman No. 3MM paper disc, and counted using the scintillation fluid (4). The data reported are from an average of two independent duplicate experiments.

RESULTS AND DISCUSSION

Analysis of the FabZ Sequence—PfFabZ (GenBank™ accession number AF237572) encoded a protein of 230 amino acids...
with a predicted molecular mass of 26.2 kDa. As can be seen in Fig. 1A, PfFabZ is more similar to plant FabZs than to the bacterial FabZ. PfFabZ, like its counterparts from plants, has a long N-terminal sequence, which is characteristic of proteins targeted to the apicoplast (36, 37). Plasmodium and Toxoplasma FabZ sequences share 40.5% identity. In the case of Toxoplasma gondii the mature protein is predicted to start at position 79 of the protein. The residues are highly conserved in this region between P. falciparum and T. gondii FabZ. Thus, we designed primers for cloning the sequence encoding the mature protein (i.e., without the signal and transit sequence) starting at position 77 of P. falciparum FabZ.

Initial attempts to PCR amplify PfFabZ using Plasmodium DNA resulted in a product of ~800 bp (Fig. 2A). However, upon performing RT-PCR using the same primers on Plasmodium RNA, a band corresponding to ~465 bp was obtained, thus demonstrating the presence of an intron in the open reading frame (Fig. 2B). Indeed upon careful examination of the full-length gene, typical intron processing sites were observed. We therefore cloned the RT-PCR product encoding the mature FabZ in pET28a(+ vector).

Expression and Purification of FabZ—The mature PfFabZ (without the signal and transit sequence) was expressed in E. coli BL21(DE3) cells as a fusion protein with an N-terminal His tag. The removal of the mature protein of an additional 10 residues, which do not contribute to enzyme function, following the transit peptide sequence led to an increase in the protein yield from 5 to 15 mg/liter of culture (truncated PfFabZ). The protein was purified on a Ni-NTA affinity column to homogeneity as seen in Fig. 2C. The purified truncated protein on SDS-PAGE yields a monomeric molecular weight of M_r 17,000 ± 1500. PfFabZ eluted at an elution volume of 17.5 ml in a Superdex 200 column corresponding to an apparent molecular weight of M_r 34,200 ± 2500. Thus, PfFabZ exists as a dimer under these conditions (2 mg/ml PfFabZ in 20 mM Tris, 500 mM NaCl, pH 7.5) (Fig. 2D). The protein yielded a radius of gyration of 6.79 ± 0.15 nm confirming that it exists as a dimer in solution (Fig. 2E).

Kinetic Characterization of FabZ—PfFabZ was characterized in vitro by spectrophotometric as well as HPLC assay. FabZ catalyzes the reversible dehydration of a β-hydroxyacyl-ACP to an enoyl-ACP as depicted in Reaction 1 below.

**Scheme II. Synthesis of inhibitors of PfFabZ.** A. 4,4,4-trifluoro-1-(4-nitrophenyl)butane-1,3-dione (NAS-21). B. 1-(4-methoxyphenyl)ethanone[(4-trifluoromethyl)pyrimidine-2-yl]hydrazone (NAS-75) and 1-(4-methyl)phenylethanone [(4-trifluoromethyl)pyrimidine-2-yl]hydrazone (NAS-79). C. 4-chloro-2-[[5-chloroquinolin-8-yl]oxy]phenol (NAS-91). MTBE, methyl tert-butyl ether; NMP, N-methylpyrrolidinone; r.t., room temperature.
reverse reaction \( (k_r/k_f) \) as calculated from spectrophotometric assay is 1:7, reiterating the fact that the equilibrium of the PfFabZ reaction lies toward the hydration reaction. Indeed it has been shown earlier that the equilibrium of the dehydratase-catalyzed reaction of \( E. coli \) lies toward \( \beta \)-hydroxyacyl-ACP; however, this activity of dehydratase is critical to supplying trans-2-acyl-ACP to FabI, which pulls each cycle of elongation to completion (11, 15).

**Homology Modeling of PfFabZ**—The sequence of \( P. falciparum \) FabZ was BLAST searched and compared for its similarity with FabZ from other organisms using ClustalW (38). As shown in Fig. 1A the sequence of PfFabZ is most similar to the corresponding enzyme from plants and bacteria consistent with its evolutionary linkage to a photosynthetic bacterium and its location in the apicoplast of the parasite. We also note that in PfFabZ, His-133 and Ghu-147 are conserved with FabZ from all the organisms reported till date. These residues are perhaps involved in its enzyme activity as they occupy positions similar to the catalytically active His-70 and Asp-84 of \( E. coli \) FabA. So far FabZ from only a few sources has been characterized, and the structure of the enzyme has not been reported from any organism. PfFabZ shares 70% amino acid sequence similarity with \( E. coli \) FabA, whose structure and function is well studied (Fig. 1B). FabA performs dehydratase as well as isomerase activity, and the two residues involved in the activity are His-70 and Asp-84 (39). While histidine is conserved in the two cases, the other residue, a glutamic acid, occupies a position in FabZ identical to that of an aspartic acid of FabA. Much of the 21% identity between PfFabZ and \( E. coli \) FabA is localized in the active site region, and the two protein sequences have 70% overall similarity. An examination of the homology-modeled structure of PfFabZ shows that the sequence of PfFabZ is consistent with the \( \alpha + \beta \) “hot dog” fold. The modeled structure of PfFabZ enables us to propose two identical active sites made by the residues from different subunits at the dimer interface, and this is extrapolated from the similar feature of the homologous \( E. coli \) FabA (39). Thus, PfFabZ is a physiological dimer. According to the PfFabZ model, the dimensions of the monomer are \( 55 \times 35 \times 35 \) Å, and those of the dimer are \( 64 \times 35 \times 47 \) Å. This correlates with the dynamic light scattering data, which give a radius of gyration of 6.79 ± 0.15 nm for the dimer of native PfFabZ indicating that it exists as a dimer in solution. Gel filtration data are also consistent with these interpretations.

**Inhibitors of FabZ Reaction**—Due to the importance of FabZ in the cellular fatty acid biosynthesis, it is a potent target for the development of antimalarials. While inhibitors of several of the enzymes of FAS II are known and extensively studied (40, 41), including FabA, which is known for its classic susceptibility to mechanism-based inactivation by 3-decenoyl-N-acetylcytsteamine, a synthetic substrate based analog (42, 43), no inhibitor of FabZ is known to date. We started by designing compounds that have a wide range of functional groups of different series for inhibition of FabZ (Scheme II), viz (i) hydrazones having trifluoromethylpyrimidine rings as well as methoxyphenylmethyl phenyl (NAS-75 and NAS-79), (ii) diketone having para-nitrophenyl, trifluoromethyl, and an active methane group (NAS-21), which can easily tautomerize to its keto-enol form, (iii) diaryl ethers having a phenolic group (proton donor), \( sp^2 \) hybrid nitrogen in which the lone pair electron is not involved in aromatic ring (proton acceptor), and a chlorine atom, which orients the molecule for forming a complex appropriately with the enzyme. Apart from these properties, molecular geometry has also been taken into consideration as diaryl ethers are extremely planar. Also the molecule has 16 π e− clouds, which may be involved in van der Waals interactions of inhibitors with FabZ, e.g. NAS-91.

The effect of various inhibitors was estimated by spectrophotometric as well as HPLC assay. A decrease in the rate of enzyme activity was observed in the presence of both NAS-91 and NAS-21. The \( K_i \) for the inhibition of PfFabZ reaction by NAS-91 and NAS-21 was calculated as 1.31 ± 0.09 and 1.46 ± 0.12 \( \mu \)M, respectively, by Lineweaver-Burk plots (Fig. 5). We determined a \( K_i \) of 0.9 ± 0.1 and 1.2 ± 0.15 \( \mu \)M for NAS-91 and NAS-21, respectively, by the Dixon method (Fig. 6). Both the inhibitors showed competitive kinetics with respect to crotonoyl-CoA for PfFabZ. The other two compounds synthesized, NAS-75 and NAS-79, did not cause any inhibition of the PfFabZ activity. NAS-91 has one of the rings (4-chlorophenol) similar to the well known antibacterial triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) that has been shown to inhibit enoyl-ACP reductase (FabI) of \( P. falciparum \) (4, 5). However, we did not observe any inhibition of PfFabI by NAS-91 and NAS-21 even at concentrations as high as 220 \( \mu \)M (the maximum concentra-
Fluorometric Analysis of PfFabZ-Inhibitor Interactions—PfFabZ exhibited excitation and emission wavelengths of 280 and 310 nm, respectively. The addition of both NAS-91 and NAS-21 led to a decrease in the fluorescence intensity at 310 nm. The magnitude of initial rapid fluorescence decrease \( F_0 - F \) occurring upon addition of each inhibitor concentration was fitted to Equation 2 to get a calculated value of \( K_b \) for NAS-91 and NAS-21, respectively. Hence the calculated value of change in fluorescence changes. PfFabZ (22 μM) was treated with 0–8.3 μM NAS-91 (A) and NAS-21 (B) with excitation and emission wavelengths of 280 and 310 nm, respectively. The magnitude of the fluorescence decrease \( F_0 - F \) occurring upon addition of each inhibitor concentration was fitted to Equation 2 to get a calculated value of \( K_b \) for NAS-91 and NAS-21, respectively. Inset, effect of inhibitors on the fluorescence emission spectrum of PfFabZ (solid line) and in the presence of 8.3 μM NAS-91 (A) and NAS-21 (B) (dashed line).

| Table II  | Inhibition constants derived for NAS-91 and NAS-21 |
|-----------|--------------------------------------------------|
| NAS-91    | NAS-21                                           |
| \( K_a \) (Dixon plot) (μM) | 0.9 ± 0.1 | 1.2 ± 0.15 |
| \( K_a \) (Lineweaver-Burk plot) (μM) | 1.81 ± 0.09 | 1.46 ± 0.12 |
| \( K_b \) (fluorescence) (M⁻¹) | 1.6 ± 0.04 × 10⁶ | 1.2 ± 0.03 × 10⁶ |
| \( \Delta G \) (fluorescence) (kcal/mol) | -8.36 | -8.19 |
| \( K_a \) (AutoDock) (μM) | 33.4 | 34.4 |
| \( \Delta G \) (AutoDock) (kcal/mol) | -6.11 | -6.09 |

\( a \) The accuracy of calculations by AutoDock is ±2 kcal/mol.
For example, triclosan inhibits enoyl-ACP reductase (FabI) of *E. coli* at picomolar concentrations ($K_i = 38 \text{ pm}$ (44)); however, micromolar concentrations (minimum inhibitory concentration $= 0.6–1 \mu \text{M}$ (45)) are required for inhibiting the growth of bacteria in cultures. Furthermore the possibility of additional unidentified targets for these inhibitors should also be considered as has been noted for triclosan in *Bacillus subtilis* (46).

**Inhibition of Parasite Fatty Acid Synthesis**—The incorporation of [2-14C]malonyl-CoA into fatty acids by cell-free *P. falciparum* extracts was studied in the presence and absence of inhibitors (Fig. 8B). Although NAS-91 and NAS-21 inhibited this incorporation, they had different effects on the parasite fatty acid synthesis pathway. This can be attributed to their differential mode of binding to PfFabZ, or alternatively they may be affecting differentially some other enzyme of the fatty acid biosynthesis pathway. The *in situ* incorporation of [1,2-14C]acetic acid into fatty acids by *P. falciparum* was also used as an index of the fatty acid synthesis. The incorporation of [1,2-14C]acetic acid in the parasite fatty acids was reduced by 46 and 26%, respectively, in the presence of 10 μM NAS-91 and NAS-21 (Table III).

**Docking of Inhibitors with PfFabZ**—We also docked the inhibitors with PfFabZ to seek an explanation for their affinities (Fig. 9). NAS-91 and NAS-21 gave similar $K_i$ values as calculated using AutoDock, which is consistent with our experimental enzyme inhibition data (Table II). The inhibitors docked with the active site region of the enzyme are shown in Fig. 9, A and B. These complexes have the best energies of binding. These studies depict that NAS-91 blocks the entrance of the active site and localizes near residues Lys-137, Ile-139, Pro-182, and Leu-184 from one of the subunits and Lys-165, Asn-166, and Asn-167 of the other subunit. Charge group interactions might play a role in fixing the orientation of the phenolic ring of NAS-91. NAS-21 is found in the tunnel-like active site surrounded by residues His-133, Val-143, Val-177, and Trp-179 from one subunit and Glu-147′ and Leu-168′ of the other subunit.

**TABLE III**

|          | NAS-91 | NAS-21 | [1,2-14C]acetic acid incorporated $\times 10^3$ |
|----------|--------|--------|-----------------------------------------------|
| μM       |        |        |                                               |
| 100      |        |        |                                               |
| 1000     |        |        |                                               |
| 10000    |        |        |                                               |
|        |        |        |                                               |

$^a$ Dashes represent that in the specific assay, the compound was not added.
The inhibitory activity of NAS-91 is probably related to the orientation of proton donor and acceptor groups in the transform, which imparts them with an affinity for the complementary hydrophilic and hydrophobic patches, respectively, on the enzyme. In NAS-21, there is a possibility of keto-enol tautomerism, which might play a role in the inhibition of the enzyme. The poor activity of NAS-75 and NAS-79 is probably due to the fact that these molecules, apart from having the planar rings, have the sp³ hybrid tetrahedral carbon, which makes the system rigid creating steric hindrance, which in turn makes it difficult for these complexes to form the complex with the enzyme.

In conclusion, we not only report expression and molecular properties of FabZ from *P. falciparum* but also two small organic molecules that inhibit a member of the FabZ family of enzymes paving the way for the development of not only anti-malarials but also anti-infectives targeting this enzyme of type II FAS. The availability of purified PfFabZ would contribute toward a better understanding of *P. falciparum* fatty acid biosynthesis and in developing high throughput screening assays for the identification of new inhibitors as potent antimalarials.

Acknowledgments—We thank Rajesh Ganapathi and Prof. A. K. Sood for the collection of dynamic light scattering data.

REFERENCES

1. World Health Organization (1999) *World Health Rep.* 49–63
2. Sherman, I. W. (ed.) (1998) *Malaria*, American Society for Microbiology Press, Washington, D.C.
3. Asindi, A. A., Ekanem, E. E., Ibia, E. O., and Nawangawa, M. A. (1993) *Trop. Sci.* 26542–26546
4. Kapoor, M., Dar, M. J., Surolia, A., and Surolia, N. (2001) *Nat. Med.* 7, 101–108
5. Kass, L. R. (1968) *J. Biol. Chem.* 243, 1180–1189
6. Surolia, N., and Surolia, A. (2001) *Biochem. Biophys. Res. Commun.* 289, 832–837
7. Ramya, T. N. C., Surolia, N., and Surolia, A. (2002) *Bioessays* 24, 192–196
8. Heath, R. J., White, S. W., and Rock, C. O. (2002) *Appl. Microbiol. Biotechnol.* 59, 782–787
9. Heath, R. J., and Rock, C. O. (1996) *Molecular Operating Environment (MOE)* Version 2001.07, Chemical Computing Group, Inc., Montreal, Quebec, Canada
10. Trager, W., and Jenson, J. B. (1976) *Science* 193, 673–675
11. Marrakchi, H., Zhang, Y. M., and Rock, C. O. (2002) *Biochem. Soc. Trans.* 30, 3219–3228
12. Heath, R. J., and Rock, C. O. (1995) *Anal. Biochem.* 230, 262–265
13. Gabiccini, M., and Marzilli, M. (1990) *Tetrahedron* 36, 3219–3228
14. Mehlman, E. L., and Solmajer, T. (1991) *Principles of Fluorescence Spectroscopy*, pp. 44–45, Plenum Press, New York
15. DeBuysere, M. S., and Olson, M. S. (1983) *Biochemistry* 22, 832–837
16. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* 18, 2714–2723
17. Gibson, D. L., and Vyas, S. (1979) *J. Med. Chem.* 22, 171–174
18. Goodstadt, L., and Ponting, C. P. (2001) *Bioinformatics* 17, 1794–1802
19. Patarroyo, M. A. (2000) *Mol. Biochem. Parasitol.* 108, 265–272
20. Unnasch, N., Cowman, A. F., Besra, G. S., Roos, D. S., and McFadden, G. I. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 12514–12519
21. Gasteiger, J., and Marsili, M. (1980) *Nucleic Acids Res.* 7, 243–244
22. Schwab, J. M., Ho, C.-K., Li, W.-B., Townsend, C. A., and Salituro, G. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 12514–12519
23. Goodenough, D. U., and Olson, M. S. (1983) *J. Biol. Chem.* 258, 7873–7879
24. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp. 44–45, Plenum Press, New York
25. Waller, R. F., Keeling, P. J., Donald, R. G., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A. F., Besra, G. S., Roos, D. S., and McFadden, G. I. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 12512–12517
26. Trager, W., and Jenson, J. B. (1976) *Science* 193, 673–675
27. Waller, R. F., Reed, M. B., Cowman, A. F., and McFadden, G. I. (2000) *EMBO J.* 19, 1874–1882
28. DeBuysere, M. S., and Olson, M. S. (1983) *Biochemistry* 22, 832–837
29. Heath, R. J., and Rock, C. O. (1996) *Molecular Operating Environment (MOE)* Version 2001.07, Chemical Computing Group, Inc., Montreal, Quebec, Canada
Identification, Characterization, and Inhibition of *Plasmodium falciparum* β-Hydroxyacyl-Acyl Carrier Protein Dehydratase (FabZ)

Shailendra Kumar Sharma, Mili Kapoor, T. N. C. Ramya, Sanjay Kumar, Gyanendra Kumar, Rahul Modak, Shilpi Sharma, Namita Surolia and Avadhesha Surolia

*J. Biol. Chem.* 2003, 278:45661-45671.
doi: 10.1074/jbc.M304283200 originally published online August 20, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304283200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 13 of which can be accessed free at http://www.jbc.org/content/278/46/45661.full.html#ref-list-1