Sulfur Mobilization for Fe-S Cluster Assembly by the Essential SUF Pathway in the *Plasmodium falciparum* Apicoplast and Its Inhibition

Manish Charan,^a Nidhi Singh,^a Bijay Kumar,^a Kumkum Srivastava,^b Mohammad Imran Siddiqi,^a Saman Habib^a

Division of Molecular and Structural Biology, CSIR-Central Drug Research Institute, Lucknow, India^a; Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow, India^b

The plastid of the malaria parasite, the apicoplast, is essential for parasite survival. It houses several pathways of bacterial origin that are considered attractive sites for drug intervention. Among these is the sulfur mobilization (SUF) pathway of Fe-S cluster biogenesis. Although the SUF pathway is essential for apicoplast maintenance and parasite survival, there has been limited biochemical investigation of its components and inhibitors of *Plasmodium* SUFs have not been identified. We report the characterization of two proteins, *Plasmodium falciparum* SufS (PfSufS) and PfSufE, that mobilize sulfur in the first step of Fe-S cluster assembly and confirm their exclusive localization to the apicoplast. The cysteine desulfurase activity of PfSufS is greatly enhanced by PfSufE, and the PfSufS-PfSufE complex is detected *in vivo*. Structural modeling of the complex reveals proximal positioning of conserved cysteine residues of the two proteins that would allow sulfide transfer from the PLP (pyridoxal phosphate) cofactor-bound active site of PfSufS. Sulfide release from the L-cysteine substrate catalyzed by PfSufS is inhibited by the PLP inhibitor D-cycloserine, which forms an adduct with PfSufS-bound PLP. D-Cycloserine is also inimical to parasite growth, with a 50% inhibitory concentration close to that reported for *Mycobacterium tuberculosis*, against which the drug is in clinical use. Our results establish the function of two proteins that mediate sulfur mobilization, the first step in the apicoplast SUF pathway, and provide a rationale for drug design based on inactivation of the PLP cofactor of PfSufS.

The relic plastid of the malaria parasite, the apicoplast, has long been thought to be its “Achilles’ heel,” as it contains pathways of bacterial origin that are not found in the human host. The apicoplast is essential for parasite survival (1, 2). It harbors unique pathways such as the type II fatty acid biosynthesis pathway, the DOXP pathway of isoprenoid synthesis, and the heme biosynthesis pathway (3–5), that offer potential novel sites for drug intervention at different stages of the infection cycle. In addition, experimental evidence of the existence of the sulfur mobilization (SUF) pathway for biogenesis of Fe-S clusters on organellar proteins was provided by our laboratory (6), and this pathway has recently been shown to be essential for apicoplast maintenance in erythrocytic stages of *Plasmodium falciparum* (7).

Fe-S clusters are believed to be among the first catalysts in nature; they act as versatile cofactors for essential biological functions in all living organisms by serving as catalytic centers in electron transfer enzymes and functioning in redox and nonredox catalysis (8). Fe-S clusters are most commonly assembled onto apoproteins as rhombic 2Fe-2S or cubic 4Fe-4S forms with Fe ions in the cluster being coordinated by cysteinates from the protein (9). Fe-S cluster assembly involves largely conserved core pathways. In bacteria, three distinct sets of factors or enzymes comprising the NIF (nitrogen fixation), ISC (iron-sulfur cluster formation), and SUF (sulfur mobilization) pathways assemble Fe-S clusters (10). NIF and ISC are housekeeping Fe-S assembly systems for bacteria, whereas the SUF pathway functions under conditions of oxidative stress or iron starvation (11, 12). In eukaryotes, the ISC pathway is the mitochondrial assembly system, while the SUF pathway is found in plastid-containing species (13, 14).

Components of the three pathways first mobilize sulfur atoms from L-cysteines and assemble them onto scaffold components that also receive iron from iron donors. The Fe-S cluster on the scaffold is subsequently transferred to the target apoprotein via an

A-type carrier (ATC) (Fig. 1). The SUF pathway employs the cysteine desulfurase SufS and its partner SufE for release of sulfur from L-cysteine. A SufBC₂D (or SufB₂C₂) scaffold complex provides the chemical and structural environment for the assembly of Fe-S clusters. The SufC ATPase in the complex is required to bring the sulfur and iron into the scaffold protein SufB (15). The assembled Fe-S clusters on the scaffold are transferred to the ATC protein SufA, which in turn transfers them to a wide range of targets (16).

Plasmodium spp. encode the ISC and SUF pathways (8, 17), some of whose constituent proteins have recently been shown to partition to the parasite mitochondrion and apicoplast, respectively (6, 7). The 35-kb genome of the apicoplast carries the gene encoding SufB, while all of the other proteins of the SUF pathway are encoded by the nucleus. In *P. falciparum*, the targeting of SufC, SufS, and SufE to the apicoplast and the interaction of apicoplast-encoded SufB with SufC have been demonstrated (6, 7). Nucleus-encoded SufA and SufD homologues carrying apicoplast-targeting signal and transit peptide sequences have been identified in *P. falciparum* (6, 17). In *P. berghei*, a scaffold NifU homologue has been shown to target to the apicoplast but is proposed to play only an auxiliary or redundant role in Fe-S formation (18).

The essentiality of the SUF pathway for apicoplast maintenance

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Address correspondence to Saman Habib, saman.habib@gmail.com.

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FIG 1 Schematic representation of the steps involved in Fe-S cluster assembly on target apoproteins by the SUF pathway.

nance and parasite survival in erythrocytic stages, as recently confirmed from disruption of the *P. falciparum* *sufC* gene (7), identifies it as a leading potential target for antimalarial drug discovery. The absence of any known inhibitors of Suf proteins underlines the need for biochemical and structural characterization of *Plasmodium* Suf proteins that would enable target-specific drug design and testing for inhibition. We report apicoplast-specific localization and functional characterization of *PfSufS* and *PfSufE*, which mobilize sulfur in the first step of cluster biogenesis. Using an inhibitor of the PLP (pyridoxal phosphate) cofactor, we also identify an approach for inhibiting the cysteine desulfurase activity of *PfSufS*.

MATERIALS AND METHODS

Parasite culture. *P. falciparum* strains (3D7 and D10 ACP_{leader}-GFP [green fluorescent protein]) were cultured by standard methods (19). Infected red blood cells (RBCs) were maintained in RPMI 1640 supplemented with 0.5% Albumax (Invitrogen). Total RNA was isolated with TRIzol (Invitrogen), and cDNA synthesis was performed with the SuperScript III first-strand synthesis system for reverse transcription-PCR (Invitrogen).

The SYBR green assay (20) for antimalarial activity was performed with dual synchronized parasites treated with various concentrations of D-cycloserine (DCS) in ring stages at 0.5% parasitemia and 1% hematocrit. Readings were taken at 48 and 96 h on a Biotek FLX800 instrument (excitation at 485 nm, emission at 530 nm). The use of human RBCs from healthy volunteers for *P. falciparum* culture was approved by the CSIR-Central Drug Research Institute (CDRI) Institutional Ethics Committee (Human Research) (CDRI/IEC/CEM/21-07-2010). Written informed consent was obtained from voluntary donors for the use of this sample in research.

Protein expression and purification. The DNA sequences encoding the predicted processed forms of *PfSufS* (PlasmoDB [plasmodb.org] accession no. PF3D7_0716600) and *PfSufE* (PlasmoDB accession no. PF3D7_0206100) were PCR amplified with *P. falciparum* total cDNA as the template. The sequence encoding amino acids (aa) 96 to 546 of *PfSufS*

was amplified with forward (5'-CGCGGATCCGAGAAGATGAGTGAGTTTAT-3') and reverse (5'-CCCAAGCTTTTTCATTTTCATTTCATTAA-3') primers; the gene sequence encoding aa 101 to 249 of *PfSufE* was amplified with primers 5'-CGCGGATCCGATGAATACAACCTAACACCAAAATTGAAA-3' (forward) and 5'-CCCAAGCTTATTGTCCATA TTCTCAAGATATTGGTGCA-3' (reverse) carrying BamHI and HindIII restriction sites (underlined). The PCR products were cloned into the pQE-30 vector (Qiagen), and clones were confirmed by DNA sequencing. pQE30-*sufS* and pQE30-*sufE* were cotransformed with the RIG plasmid (gift from W. G. J. Hol, University of Washington) into *Escherichia coli* TG1 cells. Cultures were grown at 37°C until the A₆₀₀ reached 0.4 to 0.6. Following this, the expression of recombinant proteins His₆-*PfSufS* and His₆-*PfSufE* was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 20°C. Recombinant *PfSufS* (~53 kDa) and *PfSufE* (~18 kDa) were purified by affinity chromatography with Ni-nitrilotriacetic acid agarose (Qiagen). Proteins were eluted with 250 mM imidazole. Eluted *PfSufS* was dialyzed against a buffer containing 50 mM Tris (pH 7.5), 200 mM NaCl, and 10% glycerol, and *PfSufE* was dialyzed against a buffer containing 50 mM Tris (pH 8.0), 200 mM NaCl, and 10% glycerol. Purified proteins were stored at -80°C and quantitated with Bradford reagent (Sigma).

Generation of Abs. Antibodies (Abs) against purified recombinant *PfSufS* and *PfSufE* were raised in rabbits. Approval for animal use was given by the Institutional Animal Ethics Committee, CDRI, Lucknow, India. Abs against the antigen were purified (21) and used for Western blotting and immunofluorescence microscopy.

Mutagenesis of *PfSufS*. The *PfSufS* Cys497A mutant protein was generated with pQE30-*sufS* as the template. The QuikChange XL site-directed mutagenesis kit (Stratagene) was used to mutate the *PfSufS* gene. The mutation was confirmed by DNA sequencing. Purification of the mutant protein was carried out as for wild-type recombinant *PfSufS*.

Pulldown assay. For investigation of *in vitro* complex formation of recombinant *PfSufS* and *PfSufE*, anti-SufS Abs were cross-linked to protein A Sepharose CL4B beads (GE Healthcare) with dimethyl pimelimidate (Sigma) cross-linker (22). *PfSufS* and *PfSufE* were coincubated for 1 h at 4°C; this was followed by further incubation of the mixture with anti-SufS Ab-cross-linked beads for 12 h at 4°C. Beads were washed with wash buffer (50 mM Tris, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 0.25% bovine serum albumin [BSA]) and phosphate-buffered saline (PBS), and proteins were eluted in 1× Laemmli buffer. Proteins were separated on a 12% SDS-polyacrylamide (PA) gel, followed by Western blotting with horseradish peroxidase-tagged anti-His Ab (Calbiochem).

For coimmunoprecipitation from parasite lysate, parasites were lysed in buffer containing 50 mM Tris, 1% Triton X-100, 0.6 M KCl, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride; this was followed by preclearing with protein A Sepharose CL4B beads cross-linked to anti-*PfSufS* Ab as described in reference 23. The precleared lysate was incubated with fresh cross-linked Sepharose beads for 12 h at 4°C. The beads were then washed three times with 0.1% BSA in 1× PBS. The beads were boiled in 2× Laemmli buffer for 5 min, and the samples were loaded onto a 12% SDS-PA gel; this was followed by Western blotting with anti-*PfSufS* and anti-*PfSufE* Abs, respectively.

Chemical cross-linking. *In vivo* cross-linking was carried out with dithiobis(succinimidyl propionate) (DSP; Calbiochem), a reduction-sensitive, cell-permeating cross-linker. Cross-linking was performed as described in reference 24. Briefly, infected RBCs were pelleted at 400 × g for 15 min and washed with PBS. Cells were resuspended in PBS containing 2 mM DSP and incubated at room temperature for 30 min. The cross-linking reaction was stopped by incubation with a final concentration of 25 mM Tris (pH 7.5) for 15 min at room temperature. Parasites were released from RBC by saponin lysis, resuspended in 1× NRSB buffer (0.05 M Tris-Cl, 10% glycerol, 2 mM EDTA, 2% SDS, bromophenol blue) with increasing concentrations of dithiothreitol (DTT), and then incubated at 80°C for 5 min. The samples were electrophoresed in an SDS-PA gel and then Western blotted with rabbit anti-SufS and anti-SufE Abs.

Cysteine desulfurase assay. *PfSufS* desulfurase activity was determined by the sulfide detection method as described in reference 25. Enzymatic assays were carried out anaerobically in 100 μ l of the final reaction mixture. Reaction mixtures contained 5 μ M *PfSufS* (or *E. coli* SufS) and various molar ratios of *PfSufE* in 25 mM Tris-Cl–100 mM NaCl buffer (pH 7.4)–100 μ M PLP–2 mM DTT. Reactions were initiated by the addition of L-cysteine to a final concentration of 1 mM and allowed to proceed for 20 min. Reactions were stopped by the addition of 12 μ l of 20 mM *N,N*-dimethyl-*p*-phenylenediamine (Sigma) in 7.2 M HCl and 12 μ l of 30 mM ferric chloride (Sigma) in 1.2 M HCl. Sulfide production was quantified colorimetrically by measuring absorbance at 670 nm. Inhibition by DCS was assayed as described above, except that *PfSufS* and *PfSufE* were preincubated with various concentrations of DCS for 1 h at 4°C prior to initiation of the reaction with L-cysteine.

PLP binding assay. UV-visible absorption spectra of *PfSufS* and *EcSufS* were taken in 50 mM Tris-Cl–200 mM NaCl buffer (pH 7.5). Absorption spectra were recorded at wavelengths of 250 to 500 nm. When a sample was reduced by the addition of 2 mM sodium borohydride (NaBH_4), the spectrum was recorded 1 min after the addition of NaBH_4 to the enzyme. The UV-VIS spectrum in the presence of DCS was recorded after the incubation of *PfSufS* with 5 or 0.5 mM DCS for 3 h at 4°C.

Confocal microscopy. Immunofluorescence assays were carried out as described by Tonkin et al. (4). For mitochondrial staining, *P. falciparum* 3D7 cells were incubated with 50 ng/ml Mitotracker Red CMXRos (Invitrogen) prior to fixation. Cells were washed with PBS and fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min. After one wash with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After another PBS wash, cells were treated with 0.1 mg/ml NaBH_4 in PBS for 10 min. Cells were washed again with PBS and blocked in 3% BSA–PBS for 1 h. For *PfSufS* localization, cells were incubated overnight with rabbit anti-*PfSufS* serum (1:25 dilution) and mouse anti-*PfHU* (nucleus-encoded bacterial histone-like protein) serum (1:100 dilution) (23) at 4°C. After five washes with PBS, cells were probed with Alexa Fluor 568-tagged anti-rabbit Ab (1:1,000 dilution; Invitrogen) and Alexa Fluor 488-tagged anti-mouse Ab (1:1,000 dilution; Invitrogen) for 2 h at room temperature and allowed to settle onto poly-L-lysine-coated coverslips. For localization of *PfSufE*, *P. falciparum* D10-ACP_{leader}-GFP (26) cells were fixed as described above and parasites were probed with anti-*PfSufE* serum (1:25 dilution) and mouse anti-GFP Ab (1:100 dilution; Roche) as primary Abs and Alexa Fluor 488-tagged anti-mouse Ab (1:1,000 dilution; Invitrogen) and Alexa Fluor 568-tagged anti-rabbit Ab (1:1,000 dilution; Invitrogen) as secondary Abs. For costaining with Mitotracker Red, *P. falciparum* 3D7 cells were fixed and probed with anti-*PfSufE* serum as the primary Ab and Alexa Fluor 514-tagged anti-rabbit secondary Ab. Slides were scanned in a confocal laser scanning microscope (Zeiss LSM-510) under a 63 $\times$ oil immersion lens.

Molecular modeling. To explore the three-dimensional structures and interaction of *PfSufS* and *PfSufE*, homology models were constructed. BLASTP of the *PfSufS* and *PfSufE* sequences was performed against the sequences in the Protein Data Bank (PDB) to identify suitable templates. Crystal structures of the *E. coli* CsdB NifS homologue (1CON) and *E. coli* SufE (1MZG) were selected as the templates for *PfSufS* and *PfSufE*, respectively, on the basis of identity and query coverage. Models were generated with Modeler9v10 (27). Models were then subjected to the SAVeS server (<http://nihserver.mbi.ucla.edu/SAVES/>) for structural validation by the Ramachandran plot.

To get an insight into the *PfSufS*–*PfSufE* interaction, docking of *PfSufS* and *PfSufE* was performed with the GRAMMX server (<http://vakser.bioinformatics.ku.edu/resources/grammx/grammx/>) (28), which employs smoothed potentials, extensive refinement, and knowledge-based scoring with fast Fourier transform global search methodology to predict the structure of a protein complex.

FIG 2 Expression of recombinant *PfSufS* and *PfSufE* and their detection in the parasite. (A) Coomassie-stained SDS-PAGE of purified recombinant *PfSufS* expressed as a 6 $\times$ His-tagged protein in *E. coli* (i) and detection of the protein in *P. falciparum* lysate (ii) by Western blotting with preimmune serum (Pre-I) and anti-SufS serum (I). (B) Purified recombinant *PfSufE* seen in a Coomassie-stained SDS-PAGE gel (i) and detection of the protein in parasite lysate by Western blotting with anti-*PfSufE* Ab (I) (ii).

RESULTS AND DISCUSSION

PfSufS and *PfSufE* are processed and targeted to the apicoplast.

Homologs of bacterial SufS and SufE are encoded by the *Plasmodium* nuclear genome (PlasmoDB accession no. PF3D7_0716600 and PF3D7_0206100, respectively). Although these proteins have low overall sequence identity with their *E. coli* counterparts, they carry conserved domains and residues that are critical for cofactor binding and function (see Fig. S1 and S2 in the supplemental material). *PfSufS* contains a PLP-binding motif (SGHK) with a conserved Lys residue that forms an internal aldimine with PLP; other residues that interact with PLP in the internal aldimine and substrate-bound external aldimine forms (29) are also conserved (see Fig. S1). *PfSufS* also has the conserved Cys₄₉₇ residue (corresponding to *E. coli* Cys₃₆₄) in a typical consensus region, RXGH-HCA, found in NifS-like proteins classified as group II cysteine desulfurases (30). On the basis of sequence alignment (see Fig. S2), *PfSufE* is predicted to have a 102-aa N-terminal apicoplast-targeting leader sequence (31) and carries a conserved Cys₁₅₄ residue (corresponding to Cys₅₁ in *E. coli* SufE) that is known to receive sulfur from SufS in the bacterial system (32).

In order to initiate biochemical characterization of *PfSufS* and *PfSufE* homologs, we carried out recombinant expression of their predicted processed forms in *E. coli*. Expression of the predicted processed form of *PfSufS* (aa 60 to 546) from a codon-optimized synthetic gene with an N-terminal 6 $\times$ His or maltose-binding

FIG 3 Immunofluorescence localization of *PfSufS* and *PfSufE*. (A) Immunofluorescence assay of *P. falciparum* 3D7 cells with anti-*PfSufS* Ab with anti-*PfHU* Ab as an apicoplast marker (i) or Mitotracker Red (ii) indicates targeting of *PfSufS* to the apicoplast. (B) Colocalization of *PfSufE* and apicoplast-targeted GFP observed in the *P. falciparum* D10 ACP_{leader}-GFP cell line with anti-*PfSufE* and anti-GFP Abs (i). No overlap of *PfSufE* signal was observed with Mitotracker Red in *P. falciparum* 3D7 cells (ii), indicating apicoplast localization of *PfSufE*. DIC, differential interference contrast; DAPI, 4',6-diamidino-2-phenylindole.

protein tag was attempted. However, the protein was highly unstable and a full-length form could not be purified. A functional form of *PfSufS* retaining the essential conserved motifs and covering aa 96 to 546 was subsequently expressed as a 6×His-tagged protein, purified, and used in subsequent experiments (Fig. 2Ai). Some lower-molecular-weight degraded forms of the protein were also obtained in addition to major full-length 53-kDa *PfSufS*. *PfSufE* (aa 101 to 249, ~20 kDa) was expressed as a soluble protein with an N-terminal 6×His tag (Fig. 2Bi). Polyclonal Abs against purified *PfSufS* and *PfSufE* were raised in rabbits and used to detect the proteins in *P. falciparum* lysates. A 61.5-kDa band corresponding to the size expected for a signal-cleaved form and a 56-kDa band corresponding to the expected size of signal- and transit-cleaved *PfSufS* were detected with the anti-*PfSufS* serum in a Western blot assay (Fig. 2Aii). The anti-*PfSufE* serum also detected a major 18-kDa band corresponding to the processed *PfSufE* protein and a 27-kDa band that would represent the signal-cleaved form. Two other bands that might represent degraded or intermediate cleaved forms of the 27-kDa protein were also detected. These results demonstrate that the two proteins predicted to play a role in sulfur mobilization in the SUF pathway are expressed in the parasite.

The subcellular localization of *PfSufS* and *PfSufE* in the para-

site was investigated by immunofluorescence assays with anti-*PfSufS* and anti-*PfSufE* serum. *PfSufS* colocalized with *PfHU*, an apicoplast DNA condensation protein (23), and no overlapping signals for *PfSufS* were seen with the mitochondrial marker Mitotracker Red in *P. falciparum* 3D7 cells (Fig. 3A). *PfSufE* was localized in the *P. falciparum* D10-ACP_{leader}-GFP line that targets GFP to the apicoplast (26). Specific *PfSufE* signals overlapping apicoplast-targeted GFP were observed, and no *PfSufE* signal overlap was seen with Mitotracker Red (Fig. 3B). The localization of *PfSufS* and *PfSufE* exclusively to the apicoplast was thus confirmed. Localization of these two proteins to the apicoplast has also recently been demonstrated with a C-terminal GFP tag (7). Unlike *Arabidopsis thaliana*, where SufE is dually localized to the plastid and mitochondria (33), *P. falciparum* SufE is targeted exclusively to the apicoplast. As the apicoplast localization of the *PfSufC* ATPase and its interaction with apicoplast-encoded scaffold protein *PfSufB* have been reported earlier (6), we conclude that the first two steps of the SUF pathway, namely, sulfur mobilization and Fe-S assembly on the scaffold, are present in the apicoplast. The targeting of the third predicted scaffold component *PfSufD* and the Fe-S transfer proteins *PfSufU* and *PfSufA* remains to be confirmed.

The cysteine desulfurase activity of *PfSufS* is enhanced by *PfSufE*. Cysteine desulfurases are classified as class V aminotrans-

FIG 4 *PfSufS* is a PLP cofactor-dependent desulfurase. (A) UV-VIS absorption spectrum of *PfSufS* with and without treatment with NaBH₄. The inset shows the effect of NaBH₄ on the absorption spectrum of *E. coli* SufS. The 420-nm peak of *PfSufS*-bound PLP shifts to a 335-nm peak of reduced PLP after treatment with NaBH₄. (B) Enhancement of cysteine desulfurase activity of *PfSufS* with increasing concentrations of *PfSufE*. All of the reaction mixtures except the control (20 μM *PfSufE* alone) contained 5 μM *PfSufS*. (C) Effect of the *PfSufS* Cys₄₉₇Ala mutation on sulfide release in the presence of *PfSufE*.

ferases of fold type 1 (34). They are PLP-dependent enzymes with PLP bound to a Lys residue in a highly conserved HK motif that is also found in *PfSufS*. The binding of PLP to concentrated purified *PfSufS* was indicated by its light yellow color, which is typical of PLP-binding enzymes. The UV-VIS absorption spectrum of purified recombinant *PfSufS* displayed a peak centered around 420 nm in addition to the major protein peak at 280 nm. The 420-nm peak is indicative of bound pyridoxal in an aldimine linkage in the enzyme (Fig. 4A). Addition of the reducing agent sodium borohydride to *PfSufS* resulted in reduction of the 420-nm peak and the appearance of a peak close to 335 nm (reduced PLP), an observation consistent with its known effect on PLP-binding proteins (30) (Fig. 4A). A similar shift in the UV-VIS absorption spectrum in the presence of sodium borohydride was also seen with *E. coli* SufS, which is known to be a PLP-dependent enzyme (35) (Fig. 4A, inset).

The ability of recombinant *PfSufS* to function as a cysteine desulfurase was measured in the presence of the L-cysteine substrate (25). *PfSufS* alone exhibited very low cysteine desulfurase activity (Fig. 3B). The activity of *PfSufS* was greatly enhanced by *PfSufE* (up to ~17-fold) in a concentration-dependent manner (Fig. 4B). *PfSufE* by itself had no cysteine desulfurase activity. Even though *PfSufE* has very low sequence identity with *E. coli* SufE (~23%), it could enhance the activity of *E. coli* SufS (see Fig. S3 in the supplemental material), indicating that critical interactions between the two proteins are conserved in *Plasmodium* and bacteria. Our results demonstrate that the *P. falciparum* apicomplast-targeted *PfSufS* homolog is indeed a cysteine desulfurase

that requires *PfSufE* for efficient mobilization of sulfur from L-cysteine.

The sulfane sulfur released from the L-cysteine substrate by cysteine desulfurases is transferred to generate a protein-bound persulfide intermediate at the SufS active site (32), and the stimulatory effect of SufE is dependent on transfer of the sulfur atom from the active site persulfide to a conserved cysteine of SufE (25). The SufS active site cysteine in *E. coli* is Cys₃₆₄, which corresponds to Cys₄₉₇ in *PfSufS*. In order to confirm the role of this residue in cysteine desulfurase activity, we generated a *PfSufS* Cys₄₉₇Ala mutant protein. The mutant protein *PfSufS* was unable to mobilize sulfur, and no desulfurase activity could be detected in the presence of *PfSufE*, thus establishing that Cys₄₉₇ is the critical residue involved in sulfur mobilization from the cysteine substrate (Fig. 4C).

***PfSufS*-*PfSufE* complexation is detected in the parasite.** The interaction of recombinant *PfSufS* and *PfSufE* was investigated by a pulldown assay with beads cross-linked to anti-*PfSufS* Ab (Fig. 5A). After incubation and washing, *PfSufE* was recovered together with *PfSufS* (Fig. 5A, lane 2) and did not bind the beads in the absence of its interacting partner (Fig. 5A, lane 3). *In vitro* complexation of the two proteins was thus observed.

Immunoprecipitation from parasite lysates with beads cross-linked to anti-*PfSufS* Ab resulted in the coprecipitation of *PfSufS* and *PfSufE*, as revealed by Western blotting (Fig. 5B). Formation of the *PfSufS*-*PfSufE* complex in the parasite was confirmed by chemical cross-linking by DSP, followed by reduction of the cross-linked products by DTT. A cross-linked complex (~74 kDa) and

FIG 5 *PfsufS* and *PfsufE* complex *in vitro* and *in vivo*. (A) Coimmunoprecipitation of recombinant *PfsufS* and *PfsufE* with *PfsufS* Abs cross-linked to protein A Sepharose beads, followed by Western blotting with anti-His Ab. Arrows indicate ~53-kDa *PfsufS* and ~20-kDa *PfsufE* (lane 2). No signal was observed in beads cross-linked with preimmune serum (lane 1) or in the presence of *PfsufE* alone (lane 3). Lane 5 is purified *PfsufE* used as size marker. (B) Coimmunoprecipitation of *PfsufE* with *PfsufS* from parasite lysates with beads cross-linked to anti-*PfsufS* Ab. Coprecipitated *PfsufS* (i) and *PfsufE* (ii) were detected in Western blot assays with anti-*PfsufS* and anti-*PfsufE* Abs, respectively. (C) Parasite proteins were cross-linked *in vivo* by DSP and then treated with increasing concentrations of DTT to break the complex(s). SDS-PA gels of the samples were probed with anti-*PfsufE* Ab (i) or anti-*PfsufS* Ab (ii) in Western blot assays to detect the complex and released proteins.

its DTT-mediated breakage products were detected by Western blotting with anti-*PfsufE* and anti-*PfsufS* Abs (Fig. 5C). The intensity of this complex decreased slightly in the presence of DTT (up to 200 μ M) with increasing intensity of released *PfsufE* (Fig. 5Ci) or *PfsufS* (Fig. 5Cii). An ~37-kDa band, possibly representing a degraded product of *PfsufS*, was also detected in addition to the 56-kDa processed form of the protein (Fig. 5Cii). The coimmunoprecipitation of *PfsufS* and *PfsufE* from parasite lysates, as well as the size of the DSP-cross-linked complex (74 kDa), suggests that monomers of *PfsufS* (56 kDa) and *PfsufE* (18 kDa) interact within the parasite apicoplast to mobilize sulfur release from L-cysteine.

Structural modeling of the PfsufS-PfsufE interaction. Homology modeling of *PfsufS* and *PfsufE* was carried out with crystal structures of *E. coli* CsdB/SufS (PDB accession no. 1C0N) and *E. coli* SufE (PDB accession no. 1MZG) as the templates. Processed *PfsufS* and *PfsufE* have only 30 and 23% identity, respectively, with their *E. coli* homologs (see Fig. S1 and S2 in the supplemental material). Even with its very low identity, *PfsufE* was modeled very well on the template (see Fig. S4), with Ramachandran plot analysis indicating 96.1, 3.9, 0.0, and 0.0% of its residues in the core, allowed, generously allowed, and disallowed regions, respectively. Although major structural folds of *PfsufS* were also conserved, the large 66-aa insertion in its C-terminal half could not be modeled as it lacked identity with any known protein and appeared as a loop in the structure (see Fig. S4). Overall, the modeled

PfsufS structure had 86.6, 9.9, 2.4, and 1.1% of its residues in the core, allowed, generously allowed, and disallowed regions in Ramachandran plot analysis. Structural superimpositions with templates showed root mean square deviations of 0.598 and 0.387 Å for SufS and SufE, respectively.

If the sulfur atom of the persulfide on Cys₄₉₇ of *PfsufS* (corresponding to Cys₃₆₄ of *EcSufS*) has to be transferred to Cys₁₅₄ of *PfsufE* (corresponding to Cys₅₁ of *EcSufS*) through a direct transpersulfuration reaction (32), the two cysteines of the interacting partners must be in proximity in the *PfsufS*-*PfsufE* complex. Since the structure of a SufS-SufE complex is not available, we predicted the binding interaction between *PfsufS* and *PfsufE* by protein-protein docking with the GRAMM-X docking server. Fifty possible solutions for the *PfsufS*-*PfsufE* interaction were generated. One of the docked complexes was selected on the basis of predicted proximity of the critical cysteine residues of *PfsufS* and *PfsufE* and maximal representation in the solutions (Fig. 6). Cys₄₉₇ of *PfsufS* and Cys₁₅₄ of *PfsufE* are present in the protein-protein interface in the docked solution. The distance between the sulfur atoms of the two cysteines is 8.477 Å, which would be further reduced by the additional sulfur atom of the persulfide on the *PfsufS* cysteine, thus making direct transpersulfuration to *PfsufE* plausible.

SufS from bacteria is a homodimeric enzyme with a well-separated and distinct active site in each subunit with some residues of the other subunit contributing to the active site (30, 36). The

FIG 6 Structural model of *PfSufS*-*PfSufE* interaction. (A) Protein docking with the GRAMMX server identified a complex with proximal positioning of the critical cysteine residues of the interacting proteins. A large insertion in *PfSufS* cannot be modeled and appears as a loop. (B) Enlarged portion of panel A that shows the active site and the *PfSufS*-*PfSufE* interface. The PLP cofactor is in black.

size of the complex obtained by *in vivo* cross-linking in *P. falciparum* suggests that apicomplast *PfSufS* is a monomer that interacts with *PfSufE*. Recombinant *PfSufS* was also purified as a monomer, and a dimeric form was not detected in gel filtration assays (data not shown). The presence of the large 66-aa insertion in *PfSufS* might interfere with dimerization of the protein, although this does not seem to inhibit the functionality of its active site.

A PLP-binding drug inhibits *PfSufS* and exhibits antimalarial activity. Since no inhibitors of components of the SUF pathway are known, we investigated the possibility of identifying putative inhibitors that would act either on the *PfSufS* active site or at the *PfSufS*-*PfSufE* interface. As shown in the *PfSufS*-*PfSufE* inter-

action model (Fig. 6), the protein-protein interface lacks a distinct pocket into which a putative inhibitor might be docked. Thus, the *PfSufS* active site that includes the bound PLP cofactor was considered next. Inhibitors of PLP-dependent enzymes that form adducts with the cofactor have been used as irreversible inhibitors of enzyme activity (37). One such inhibitor, DCS, is in human clinical use as a second-line drug against *Mycobacterium tuberculosis* (38). DCS acts on enzymes with PLP fold type 1 (aspartate aminotransferase family) (37, 39, 40). Since cysteine desulfurases are PLP fold type 1 proteins (41), we explored the effect of DCS on PLP interactions in *PfSufS*. A reduction of the 420-nm peak of the internal aldimine between PLP and Lys291 (corresponding to

FIG 7 DCS inhibits *PfSufS* activity. (A) UV-VIS absorption spectrum of *PfSufS* in the presence of DCS showing a reduction in the 420-nm bound PLP peak accompanied by the appearance of a peak at 320 nm representing a PLP-DCS adduct. (B) Effect of increasing concentrations of DCS on cysteine desulfurase activity of *PfSufS*. The IC_{50} of DCS was determined to be $29.2 \pm 2.9 \mu M$.

Lys226 of *E. coli*) was seen in the UV-VIS spectrum of *PfSufS* in the presence of DCS (Fig. 7A). The reduction of the 420-nm peak was accompanied by increased absorbance at ~ 320 nm that corresponds to the peak expected for the PLP-DCS adduct (3-hydroxy-isoxazole-pyridoxamine derivative) as reported for other PLP-dependent enzymes inhibited by DCS (39, 40). DCS is thus capable of forming an adduct with the PLP cofactor bound to *PfSufS*.

The effect of DCS on the cysteine desulfurase activity of recombinant *PfSufS* was investigated. Dose-dependent inhibition of *PfSufS* activity by DCS was observed in the presence of *PfSufE*. The 50% inhibitory concentration (IC_{50}) was calculated to be $\sim 29 \mu M$ (Fig. 7B). The antimalarial action of DCS in *P. falciparum* blood stage parasites was assayed by SYBR green whole-cell screening (20). Compounds that exhibit apicoplast-specific action are known to cause a delayed-death effect in which parasite growth is inhibited primarily in the second cycle of infection (42, 43). The IC_{50} of DCS for *P. falciparum* strains was thus determined for the first and second infection cycles (at 48 and 96 h posttreatment). The IC_{50} s of DCS for the growth of *P. falciparum* 3D7 were ~ 62 and $68 \mu M$ in the first and second cycles, respectively (Table 1). The IC_{50} s of DCS for chloroquine-resistant *P. falciparum* K1 were determined to be ~ 62 and $71 \mu M$ in first and second cycles. These values are close to the reported IC_{50} of DCS for *M. tuberculosis* H37Rv culture growth ($58 \mu M$) (44).

The inhibition of *PfSufS* by DCS provides evidence for the possibility of designing inhibitors targeting the PLP moiety of this important enzyme of the apicoplast Fe-S biogenesis pathway. The absence of a delayed-death effect upon DCS treatment of blood-stage parasites suggests that the drug also has additional, nonapicoplast targets in the parasite. There are more than a dozen PLP-dependent enzymes in *P. falciparum* (<http://priweb.cc.huji.ac.il/malaria/maps/vitaminB6metpath.html>) at least eight of which

are fold type 1 enzymes that could also serve as targets of DCS (see Table S1 in the supplemental material); these include *PfIscS*, the cysteine desulfurase of the ISC pathway of the parasite mitochondrion. However, *PfSufS* would be a major target of DCS *in vivo*, as also supported by the fact that the IC_{50} for parasite growth is higher than the IC_{50} for *PfSufS* desulfurase activity.

The apicoplast SUF pathway is believed to play a critical role in parasite biology by mediating the assembly of Fe-S clusters on target proteins that include ferredoxin (Fd)/ferredoxin reductase (FNR), lipoic acid synthase (LipA), (dimethylallyl)adenosine tRNA methylthiotransferase (MiaB), and the last two enzymes of the nonmevalonate DOXP pathway of isoprenoid biosynthesis [(E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (IspG) and (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase (IspH)] (45). The antimalarial effect of inhibition of Fe-S assembly on target proteins in the apicoplast has been recently demonstrated through the generation of dominant negative SufC mutant parasites (7). These parasites lost the apicoplast and the organellar genome and were viable only when supplemented with isopentenyl pyrophosphate, an essential product of the apicoplast DOXP pathway that supports parasite survival in blood culture even when the apicoplast is lost (46). Our results demonstrate that it is possible to inhibit the apicoplast SUF pathway with compounds targeting the PLP cofactor of its constituent cysteine desulfurase. Unlike its enantiomer L-cycloserine, DCS is a natural product with low toxicity in humans (38). Other PLP inhibitors with demonstrated effects on PLP cofactor-dependent target enzymes in other organisms (37) can also be tested for inhibition of *Plasmodium* SufS *in vitro* and as well as for their antimalarial activity.

The SUF pathway is not the default pathway for Fe-S biogenesis in bacteria, where the ISC and NIF pathways play more significant roles and SUF proteins are activated only under conditions of stress. This redundancy of the SUF pathway in bacteria is abolished in *Plasmodium* spp., where ISC and SUF components are partitioned to the mitochondrion and apicoplast, respectively, thus conferring all Fe-S biogenesis function on critical apicoplast targets to the SUF pathway. In addition, the SUF pathway is absent from humans, making its components attractive targets for inhibitor identification. The design of novel chemical inhibitors and

TABLE 1 IC_{50} of DCS for the first and second infection cycles

Time (h)	Avg $IC_{50} \pm SD$ (μM)	
	<i>P. falciparum</i> 3D7	<i>P. falciparum</i> K1
48	62.4 ± 0.9	62.2 ± 5.2
96	67.9 ± 1.5	71.16 ± 0.3

generation of suitable assay systems with recombinant *Plasmodium* SUF proteins and their complexes is the obvious next step.

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