Structure of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase–dihydropteroate synthase from
Plasmodium vivax sheds light on drug resistance

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The genomes of the malaria-causing Plasmodium parasites encode a protein fused of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK) and dihydropteroate synthase (DHPS) domains that catalyze sequential reactions in the folate biosynthetic pathway. Whereas higher organisms derive folate from their diet and lack the enzymes for its synthesis, most eubacteria and a number of lower eukaryotes including malaria parasites synthesize tetrahydrofolate via DHPS. Plasmodium falciparum (Pf) and Plasmodium vivax (Pv) HPPK–DHPSs are currently targets of drugs like sulfadoxine (SDX). The SDX effectiveness as an antimalarial drug is increasingly diminished by the rise and spread of drug-resistant mutations. Here, we present the crystal structure of PvHPPK–DHPS in complex with four substrates/analogs, revealing the bifunctional PvHPPK–DHPS architecture in an unprecedented state of enzymatic activation. SDX's effect on HPPK–DHPS is due to 4-amino benzoic acid (pABA) mimicry, and the PvHPPK–DHPS structure sheds light on the SDX-bonding cavity, as well as on mutations that effect SDX potency. We mapped five dominant drug resistance mutations in PvHPPK–DHPS: S382A, A383G, K512E/D, A553G, and V585A, most of which occur individually or in clusters proximal to the pABA-binding site. We found that these resistance mutations subtly alter the intricate enzyme/pABA/SDX interactions such that DHPS affinity for pABA is diminished only moderately, but its affinity for SDX is changed substantially. In conclusion, the PvHPPK–DHPS structure rationalizes and unravels the structural bases for SDX resistance mutations and highlights architectural features in HPPK–DHPSs from malaria parasites that can form the basis for developing next-generation anti-folate agents to combat malaria parasites.

Malaria remains a central cause of morbidity and mortality in humans. The malaria parasites Plasmodium falciparum (Pf) and Plasmodium vivax (Pv) infect >400 million people and result in ~0.3 to ~0.4 million annual deaths worldwide (1). Malaria remains a constant public health threat because of the emergence of drug-resistant strains across endemic regions (2). Despite increased drug resistance, several anti-malarial drugs are still used clinically for the treatment of malaria infection (3). Therefore, the world health community needs to continually discover both new drug targets and novel chemical scaffolds. Tetrahydrofolate is an essential cofactor that is vital for metabolic reactions involving one-carbon transfer (4). Most notably, it is required for the synthesis of nucleic acid precursors like purines and thymidine and for methionine, glycine, and pantothenate (5). Higher organisms derive folate from their diet and lack the necessary enzymes for folate synthesis, but almost all eubacteria and a number of lower eukaryotes including malaria parasites synthesize tetrahydrofolate (5–7). The malaria parasite genomes encode fused 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase (HPPK) and dihydropteroate synthase (DHPS) domains (Fig. 1A) that perform sequential reactions wherein HPPK catalyzes transfer of pyrophosphate from ATP to 6-hydroxymethyl-7,8-dihydropterin (DHP) resulting in 6-hydroxymethyl-7,8-dihydropterinpyrophosphate (DHPPP) (8, 9). Subsequently, DHPS acts as a crucial convergence point in the folate pathway and catalyzes the condensation of 4-aminobenzoic acid (pABA) and DHPPP to form the intermediate 7,8-dihydropteroate (Fig. 1B) (10). 7,8-Dihydropteroate is then fed into the folate synthesis path-

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The atomic coordinates and structure factors (code SZ79) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: Pf, P. falciparum; Pv, P. vivax; HPPK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; RMSD, root mean square deviation; SDX, sulfadoxine; pABA, 4-amino benzoic acid; DHP, 6-hydroxymethyl-7,8-dihydropterin; DHPPP, 6-hydroxymethyl-7,8-dihydropterinpyrophosphate; SP, SDX with pyrimethamine; ASU, asymmetric unit; PPPP, 6-hydroxymethyl pterindiphosphate; AMPCPP, adenosine 5′-(α,β-methylene)-diphosphate; MR, molecular replacement; TIM, triosephosphate isomerase; PDB, Protein Data Bank.
way where it is converted to 7,8-dihydrofolate by the enzyme dihydrofolate synthase and subsequently to tetrahydrofolate by the enzyme dihydrofolate reductase (Fig. 1B). The folate pathway is therefore an ideal target for anti-infectives and has been utilized for many decades (11).

Sulfonamides target a key enzyme in folate biosynthesis pathway viz. DHPS (12). The aryl amine moiety of sulfa drugs forms a dihydropteroate-like product with DHPPP that is impotent toward undergoing subsequent dihydrofolate synthesis (13, 14). Sulfa drugs have remained important clinical agents since they were first discovered in the 1930s (15), but their efficacy has been severely impacted by drug resistance that began to emerge shortly after they were first introduced (16, 17). Despite increased resistance, sulfadaxine (SDX) is still used in combination with pyrimethamine (SP) to treat malaria (18, 19). Pyrimethamine inhibits the enzyme dihydrofolate reductase in the folate biosynthesis pathway, whereas SDX stalls DHPS activity; because of their synergistic effect, these two drugs (SP) are more effective in combination than either drug used alone (18, 19). SP is used to treat chloroquine-resistant Pf malaria and is the only drug combination recommended by World Health Organization for intermittent preventive treat-
ment of infants as an additional malaria control in high transmission areas of sub-Saharan Africa (19–22). Increasing resistance toward SDX is therefore alarming and calls for the development of a new generation of anti-folates that are less susceptible to resistance generation but that retain the enzymatic target of DHPS.

Here, we present the crystal structure of \textit{Pv}HPPK–DHPS in complex with its four substrates/analogs. The structure contains residues from 1 to 717, is dimeric, and reveals the juxtaposition of both HPPK and DHPS domains that are connected by a linker segment. We have mapped the conserved \( p \)ABA-binding residues and sites of vital mutations that tune the specificity and affinity of \( p \)ABA, as well as the drug SDX. This work will enhance our understanding of molecular mechanisms used by \textit{Pv}HPPK–DHPS to develop anti-folate drug resistance and provides a new focus for development of novel anti-malarial agents.

\textbf{Results}

\textbf{Crystal structure of \textit{Pv}HPPK–DHPS}

\textit{Plasmodium} parasite genomes encode fused genes for HPPK and DHPS. We expressed recombinant full-length \textit{Pv}HPPK–DHPS consisting of 717 residues and crystallized it (P2\(_1\) system with six molecules in asymmetric unit (ASU)). Each \textit{Pv}HPPK–DHPS chain that was traced has 600 of 717 residues along with disordered and missing loops. The six molecules of HPPK–DHPS in the ASU are designated A to F that form three biological dimers called AB, CD, and EF. The three dimers are similar, and their root mean squared deviations (RMSDs) for C\(_\alpha\) atoms are generally <0.5 Å; they differ mostly in their long flexible loop regions. The N-terminal 10 residues and loop residues 55–80, 145–160, 189–202, 320–435, and 588–660 are disordered in all six molecules of HPPK–DHPS. The overall fold of the \textit{Pv}HPPK–DHPS domains is similar to their known homologs. The average B-factor for molecules A, C, and D is ~37 Å\(^2\), whereas for the other three molecules B, E, and F it is ~51Å\(^2\). Therefore, from hereon the structural analyses discussed are based on the CD dimer (Fig. 2A). The RMSD between the \textit{Pv}HPPK–DHPS and known HPPK and DHPS structures is <1 Å for the overlapping ferredoxin fold (C\(^\alpha\) atoms 65–95) and the triosephosphate isomerase TIM barrel core (C\(^\alpha\) atoms 165–195). The \textit{Pv}HPPK–DHPS was crystallized in the presence of 6-hydroxymethylpterin-diphosphate (PtPP), pterin, the ATP analog AMPCPP, and \( p \)ABA (Fig. 2A). The electron densities for bound ligands are clear for the monomeric chains of \textit{Pv}HPPK–DHPS (Fig. 2B and C). The electron densities for whole AMPCPP or terminal phosphate groups of the AMPCPP are weak in monomers B, D, E, and F, possibly because of their poor occupancies. The present crystal structure therefore represents a holo form of the \textit{Pv}HPPK–DHPS enzyme and will be discussed below in this light.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Crystal structure of \textit{Pv}HPPK–DHPS. A, dimeric \textit{Pv}HPPK–DHPS where bound substrate/analogs in both HPPK and DHPS domains are shown as molecular surfaces. HPPK (cyan), DHPS (yellow), and the linker regions (blue) are marked. The bound substrates/analogs of pterin (maroon), AMPCPP (light green), PtPP (purple), \( p \)ABA (green), and Mg\(^{2+}\) ion (lime green) are shown as sticks and spheres. B and C, the simulated annealing composite omit map contoured at 2\( \sigma \) levels for the bound ligands where ligands and bound Mg\(^{2+}\) ion are shown as sticks and spheres, respectively.}
\end{figure}
*Pv*HPPK has a 90-residue insertion (residues 60–150) between β-strands 2 and 3 when compared with the known homologs of HPPK structures from *Escherichia coli*, *Streptococcus pneumoniae*, *Yersinia pestis* (YP), *Saccharomyces cerevisiae*, and *Francisella tularensis* (Figs. 3 and 4A) (23–28). This insertion wraps around the ferredoxin fold of *Pv*HPPK domain, and it has three α-helices: α1’a, α1’b, and α1’c. α1’a partially masks the active site of *Pv*HPPK (Fig. 4A). The loop (L3) between β-strands β5 and β6 are important for ATP binding in HPPK (Fig. 4A). In *Pv*HPPK, a 48-residue-long insertion (residues 222–260) is present in ATP-binding loop, and this insert has two α-helices (α2’a and α2’b) when compared with the known HPPK structures (Fig. 4A).

Clear electron densities in *Pv*DHPS are evident for *p*ABA and the substrate analog PtPP (Fig. 2C). In addition, well defined electron densities were observed for all the active-site loops in *Pv*DHPS because they are highly ordered (Fig. 4B). Long insertions are not found in the core-TIM barrel fold of *Pv*DHPS domain. However, a 40-residue insertion is present between strand β3 and helix α2, and this insertion has a 10-residue helix α2’ (Fig. 4B). The helix α2 of *Pv*DHPS has 26 residues in 7 turns and is up to 9 residues longer than the other known DHPS structures that have between 14 and 17 residues (Fig. 4B) (23–28). A Mg²⁺ cofactor is known to coordinate the diphosphate group within DHPPP and involves the conserved Asn342 in *Pv*HPPK–DHPS. In *Pv*DHPS, another 80-residue-long insertion is present between α-helix 7’ and α7, and this insertion contains the 8X “tandem repeat-like” sequence motif of GEGKLTN (Fig. 4B).

*Pv*HPPK–DHPS forms a dimeric assembly via C-terminal α-helices α6, α7’, α7, and α8 (Fig. 4C). In its dimeric form, the solvent-accessible surface of *Pv*HPPK–DHPS is ~51,800 Å², and the buried surface is ~3300 Å². The association of helices α6, α7’, α7, and α8 is similar to that observed in all dimeric DHPS structures, as also in the bifunctional *S. cerevisiae* DHPS–DHPS structure (23–28). The dimer interface involves only the DHPS domain structural elements. In *Pv*DHPS, the N-terminal end of the TIM barrel is capped by antiparallel β-strands, and they are conserved among *E. coli* and *Yp*, *Thermus thermophilus* and *Bacillus anthracis* DHPSs (23–28). The capped antiparallel β-sheet serves as a linker region between *Pv*HPPK and *Pv*DHPS domains. The linker β-strand (L1) forms a short antiparallel association with the β2-strand of *Pv*HPPK domain (Figs. 3 and 4D). This is very similar to *S. cerevisiae* DHPS–DHPS structure (25). The orientation of HPPK domains does not overlap on the *F. tularensis* HPPK–DHPS (FtHPPK–DHPS) structure (27), and large displacement in HPPK is observed in context of ScHPPK–DHPS (Fig. 4D) (25). This is due to the longer β-strands in linker region of ScHPPK–DHPS and shorter linker in FtHPPK–DHPS when compared with the *Pv*HPPK–DHPS.

**The molecular mechanism of sulfadoxine resistance**

Our crystal structure of *Pv*DHPS in complex with *p*ABA shows a striking state of enzymatic activation in which five *Pv*DHPS loops bind *p*ABA onto the protein surface (Fig. 5A). The substrate *p*ABA is ensconced by loops 1, 2, 5, and 6 and the 7’ helix within loop 7. The eight key residues envelope *p*ABA in *Pv*DHPS and thus form its binding site that contains Phe³⁴⁸, Ser³⁸², Ala³⁸³, Pro³⁸⁴, Gly⁵⁵¹, Phe⁵⁵², Lys⁵⁸¹, and Arg⁵⁸² (Fig. 5B). These residues are highly conserved among *Pv* and *Pv*HPPK–DHPSs (Fig. 3, highlighted in the box). In addition to the *Pv*DHPS crystal structure reported here, the crystal structure of *Yp*DHPS bound to pABA is known (28). Therefore, we compared the *p*ABA-binding residues and their conformational states in *Pv*DHPS and *Yp*DHPS. Loops 1 and 2 encapsulating *p*ABA in *Pv* and *Yp* are shown in (Fig. 5C), and they show no significant differences. In *Pv*DHPS, key residues caging *p*ABA in loop 2 are ³⁸¹SSAPY³⁸⁵, whereas in *Yp* the residues are ³⁸¹STRPG⁶⁵ (Fig. 5, C and D). Among these, it is evident that the proline in fourth position is conserved; it nestles the benzene ring of *p*ABA via a hydrophobic interaction (Figs. 5D and 6C). The two flexible loops 1 and 2 that cover *p*ABA-binding site are highly ordered in the present *Pv*HPPK–DHPS. In case of bacterial DHPS, the majority of the mutations known to confer resistance to sulfonamides are found in these two loops. However, in the case of *Pv*HPPK–DHPS, SDX point mutations do not occur in loop 1 that contributes the single *p*ABA-binding residue Phe³⁴⁸.

The *p*ABA-binding residues Phe³⁴⁸, Gly⁵⁵¹, Phe⁵⁵², Ser³⁸², Ala³⁸³, Lys⁵⁸¹, Arg⁵⁸², and Pro³⁸⁴, as well as the SDX resistance mutations are conserved among human malaria parasites *Pv* and *Pf* (Fig. 6A). Five SDX point mutations at positions S382F/A/C, A383G, K512E/M/T, A553G, and V585A have been reported in *Pv*DHPS (29–34) (Fig. 6B). These amino acid changes occur as singles (V585A or A383G), in doubles (A383G and A553G), or triply (S382A, A383G and A553G) and together account for DHPS mutations in 90% of the clinical isolates from malaria endemic regions (29–34). Our *Pv*HPPK–DHPS crystal structure shows that these resistance mutations surrounding the *p*ABA-binding site are present within loop 2 (S382F/A/C and A383G), loop 5 (Lys⁵¹²), loop 6 (Ala³⁵³), and 7’ helix in loop 7 (Val³⁸⁵) (Figs. 3 and 6B).

We collated SDX resistance data from clinical isolates of *Pf* and *Pv* and analyzed available enzyme kinetic data from two elegant published studies in context of *Pv*ABA and SDX affinities (33, 34). We assessed the fold difference of the substrate-binding constant (*Kₘ*) of *p*ABA with the inhibitory constant (*K*) of SDX as a function of the DHPS mutations from both studies (33, 34) (Fig. 6A). It is documented that single amino acid mutations of A383G and V585A do not seem to confer very high levels of SDX resistance, as shown in Fig. 6A (33, 34). We also observed that the five key mutations responsible for SDX drug resistance mostly precede or succeed the vital atomic interactions that fall within 4 Å of the *p*ABA binding pocket (Fig. 6B). The residues Ser³⁸² and Ala³⁸³ are present within loop 2 of *Pv*DHPS, and from analyses of deposited DHPS structures, it is evident that loop 2 is highly flexible and that its sequence is conserved across DHPSs. Further, in the case of *Pv*DHPS, loop 2 clearly stabilizes *p*ABA binding by contributing (Ser³⁸², Ala³⁸³, and Pro³⁸⁴) residues for *p*ABA recognition. Based on analysis of our *Pv*HPPK–DHPS crystal structure, it is evident that Ser³⁸² and Ala³⁸³ residues make intimate interactions with *p*ABA (Fig. 5B). Further, Ser³⁸² and Ala³⁸³ precede the critical Pro³⁸⁴ residue that stacks with the benzene ring of *p*ABA (Figs. 5D and 6C). These structural constraints within the *p*ABA-binding site likely explain the mutational effects of residues 382.
Crystal structure of P. vivax HPPK–DHPS enzyme

PvX
---MEDSTGTTTFRINAILFGDTKDMKCNVTLHETLHYTGRLIINSSHSTYETPVE 57
PvP01
---MEDSTGTTTFRINAILFGDTKDMKCNVTLHETLHYTGRLIINSSHSTYETPVE 57
Pf3D7
---MEITQLILESENNTIALVNLGTNRDARNRELVETALHLEVKLRTNISYETPVE 60
PfIT
---MEITQLILESENNTIALVNLGTNRDARNRELVETALHLEVKLRTNISYETPVE 60

PvX
YIVLDENENIGEVEGEPFPR3DIGWGGELPTVENSREESDIEYCEKELVEFVLIKNEF 117
PvP01
YIVLDENENIGEVEGEPFPR3DIGWGGELPTVENSREESDIEYCEKELVEFVLIKNEF 117
Pf3D7
YIVLDKESKCRKNCRKVVRHKYINQLNQLEHSKYEYNEKELIDCSEYETFYRNGKVD 120
PfIT
YIVLDKESKCRKNCRKVVRHKYINQLNQLEHSKYEYNEKELIDCSEYETFYRNGKVD 120

PvX
ESITREVESVDENEAIIKRIKNREKMMENLQSGKDKYKTYTYYFNYILTVFVYTVFVYTVTFLAM 177
PvP01
ESITREVESVDENEAIIKRIKNREKMMENLQSGKDKYKTYTYYFNYILTVFVYTVFVYTVTFLAM 177
Pf3D7
NSILKEVNHVSEQULENN1VKNDEIKMMNLSKDYKDTYSFYNYLTFLYTVFVYTVTFLAM 180
PfIT
NSILKEVNHVSEQULENN1VKNDEIKMMNLSKDYKDTYSFYNYLTFLYTVFVYTVTFLAM 180

PvX
LVILYIIEGKRESEKKQQGEGIFQNRHIDILLYFNVNLYIFEKSLIKLSEDYKJIKY 237
PvP01
LVILYIIEGKRESEKKQQGEGIFQNRHIDILLYFNVNLYIFEKSLIKLSEDYKJIKY 237
Pf3D7
LVVYIIEGKRESEKKQQGEGIFQNRHIDILLYFNVNLYIFEKSLIKLSEDYKJIKY 238
PfIT
LVVYIIEGKRESEKKQQGEGIFQNRHIDILLYFNVNLYIFEKSLIKLSEDYKJIKY 238

PvX
IHMTDSQ-------------------------------------------- 246
PvP01
IHMTDSQ-------------------------------------------- 246
Pf3D7
IKLRIENGKNDQMKVYNMRKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRNKIMRNKMKNLNLNNGRN

PvX
-----------------------------382,383-386,437
PvP01
-----------------------------382,383-386,437
Pf3D7
-----------------------------382,383-386,437
PfIT
-----------------------------382,383-386,437

PvX
FEMASDGASVIDIDQGSQSASSPLPVFYNVDYVTVEQKLLMLKLYKEEKKMLC6E6VGGGGVCA 424
PvP01
FEMASDGASVIDIDQGSQSASSPLPVFYNVDYVTVEQKLLMLKLYKEEKKMLC6E6VGGGGVCA 424
Pf3D7
FEMASDGASVIDIDQGSQSASSPLPVFYNVDYVTVEQKLLMLKLYKEEKKMLC6E6VGGGGVCA 424
PfIT
FEMASDGASVIDIDQGSQSASSPLPVFYNVDYVTVEQKLLMLKLYKEEKKMLC6E6VGGGGVCA 424

PvX
-------------------------------ASDARKNAQSSSQQGKLRKMRHDRESARLHILEN7HVLVQGGLVDVLNDISACTHNF 484
PvP01
-------------------------------ASDARKNAQSSSQQGKLRKMRHDRESARLHILEN7HVLVQGGLVDVLNDISACTHNF 484
Pf3D7
-------------------------------ASDARKNAQSSSQQGKLRKMRHDRESARLHILEN7HVLVQGGLVDVLNDISACTHNF 484
PfIT
-------------------------------ASDARKNAQSSSQQGKLRKMRHDRESARLHILEN7HVLVQGGLVDVLNDISACTHNF 484

PvX
-------------------------------514,660
PvP01
-------------------------------514,660
Pf3D7
-------------------------------514,660
PfIT
-------------------------------514,660

PvX
IKKLRKKFIVSYLTVMLJKNPQPLTLYVDLSDIVDLHVLVNLGVPYVR 544
PvP01
IKKLRKKFIVSYLTVMLJKNPQPLTLYVDLSDIVDLHVLVNLGVPYVR 544
Pf3D7
IKKLRKKFIVSYLTVMLJKNPQPLTLYVDLSDIVDLHVLVNLGVPYVR 544
PfIT
IKKLRKKFIVSYLTVMLJKNPQPLTLYVDLSDIVDLHVLVNLGVPYVR 544

PvX
-------------------------------583,641
PvP01
-------------------------------583,641
Pf3D7
-------------------------------583,641
PfIT
-------------------------------583,641

PvX
-------------------------------588,613
PvP01
-------------------------------588,613
Pf3D7
-------------------------------588,613
PfIT
-------------------------------588,613

PvX
PQ-----------------------------664
PvP01
PQ-----------------------------664
Pf3D7
PQ-----------------------------664
PfIT
PQ-----------------------------664

PvX
-------------------------------717
PvP01
-------------------------------717
Pf3D7
-------------------------------717
PfIT
-------------------------------717
and 383, because their mutation may disturb the positioning of Pro\textsuperscript{384} that is critical for stacking with the benzene ring of pABA. Intriguingly, resistance mutation residue Lys\textsuperscript{512} lies distal to the pABA-binding site, and its role in resistance generation cannot be reliably accessed via the present HPPK–DHPS structure (Fig. 6B). The A553G mutation will likely create alteration in the presentation of loop 6 that interacts with pABA. The SDX mutant residue V585A shows a 2-fold increase in the level of resistance compared with the WT \textit{Pv}DHPS (Fig. 6A); this residue is located at dimerization interface and does not seem to play a role in the binding of either the substrate or SDX (not shown). Interestingly, our \textit{Pv}HPPK–DHPS structure reveals that residues Arg\textsuperscript{582} and Asp\textsuperscript{511} make salt-bridge interactions and are conserved in \textit{Pv} and \textit{Pf} HPPK–DHPSs. It is likely that the V585A mutation results in steric hindrance with Arg\textsuperscript{582}. This may translate into diminished interactions with Asp\textsuperscript{511} and structural perturbation of pABA recognition (Fig. 6C).

**Discussion**

The X-ray structure of \textit{Pv}HPPK–DHPS presented here rationalizes the known sulfa drug resistance mutations that have arisen over the past four decades as a result of drug usage. The \textit{Pv}HPPK–DHPS structural analyses reveal an intricate dimeric assembly via a C-terminal region in the \textit{Pv}DHPS sequence. This long-awaited crystal structure of the malaria parasite’s unique bifunctional HPPK–DHPS enzyme reveals the exquisite structural subtleties involved in SDX resistance generation. It is evident that most SDX resistance mutations map proximal to the pABA-binding site, where they are likely to subtly alter the intricate atomic interactions such that enzyme affinity for pABA is only diminished moderately (up to 11-fold), but \textit{K}_i for

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**Figure 3. Structure-based sequence alignment of malaria parasite HPPK–DHPSs.** The secondary structure elements of \textit{Pv}HPPK–DHPS (blue) and \textit{Pf}HPPK–DHPS (yellow) are marked, with $\alpha$- and $\beta$-helices (cylinders) and $\beta$-strands (arrows). The pABA-binding loops (L1, L2, L5, L6, and L7) residues are in bold, underlined, and labeled. The disordered regions in the crystal structure of \textit{Pv}HPPK–DHPS are marked by black dots. The SDX resistance mutations in \textit{Pf} (green) and \textit{Pv} (blue) are numbered above the sequences. The linker region between HPPK and DHPS is highlighted in red. The pABA-binding residues are marked with stars.

**Figure 4. \textit{Pv}HPPK–DHPS domains and structural comparisons.** A and B, the domains of \textit{Pv}HPPK–DHPS are shown with secondary structural elements. The unique features/larger insertions are also labeled. C, a view of dimer interface and the participating helices $\alpha_6$, $\alpha_7$, $\alpha_7$, and $\alpha_8$. A possible tandem repeat motif is shown. D, the superposition of DHPS domain of \textit{Pv}HPPK–DHPS on \textit{Sc}HPPK–DHPS where their unique features are highlighted.
Figure 5. The pABA-binding site in PvDHPS. A, overall view of the pABA-binding region with key loops labeled. B, key residues that cage pABA in PvDHPS. C, structural comparisons between loops that surround pABA in DHPSs from Y. pestis and P. vivax. D, critical pABA-binding residues in Pv (yellow) and Yp (gray) are shown. The pABA molecule is shown as molecular surface (green) and protein residues shown as sticks (gray and yellow).
SDX is altered substantially (more than 100-fold for double/triple mutants in both \textit{Pv}/\textit{Pf} DHPSs (Fig. 6A). Thus, a structural compromise is reached in the drug resistant enzyme wherein marginal lowering in the substrate (\textit{p}ABA) affinity ($K_m \approx \sim 11$-fold) is traded for substantial reductions ($K_i \approx \sim 800$-fold; Fig. 6A) in SDX drug potency. This trick, although highly successful, also opens the enzyme for targeting via novel inhibitors that are designed based on the exact substrate envelope such that new inhibitors fit and colonize the substrate-binding site fully and that too in at least four different druggable pockets that are evident in the \textit{Pv}HPPK–DHPS structure. Indeed, inhibitors like SDX that protrude beyond the substrate envelope may encourage development of mutations that confer drug resistance. In this light, an interesting drug design approach has been applied toward potentially overcoming drug resistance in HIV by focusing on inhibitors that fit snugly in the small substrate-binding cavity of HIV protease (35, 36).

It is noteworthy that the \textit{Pv}HPPK–DHPS residues involved in recognition of pterin moiety in both domains are highly conserved. This presents yet another opportunity to target conserved motifs within \textit{Pv}HPPK–DHPS now that the crystal structure is available (37). The presented \textit{Pv}HPPK–DHPS structure indicates that sulfa-drug resistance mutations emanate from a structural compromise in the mutant drug resistance enzyme that enables rejection of the drug while minimally altering affinity for its substrate \textit{p}ABA. Although our \textit{p}ABA-bound \textit{Pv}HPPK–DHPS crystal structure can explain SDX resistance for most mutations, we feel that SDX-bound crystal structures of mutant and WT \textit{Pv}DHPSs are required for a deeper understanding of this enzyme/drug system (37). From our structural analysis of \textit{Pv}HPPK–DHPS and its mutations in context of sulfadoxine resistance, we have generated several insights including \textit{(a)} the presented structure should be exploited to identify nonsulfa drugs that do not mimic \textit{p}ABA and thus inhibit the enzyme irreversibly, \textit{(b)} the \textit{Plasmodium} HPPK domain can now be utilized for focusing on pterin-based inhibitors (38), \textit{(c)} designing drugs that target the triple mutant in \textit{Pv}HPPK–DHPS will be valuable because they can be selectively administered in regions of prevalent SDX resistance, and \textit{(d)} twin targeting of \textit{Plasmodium} HPPK and the DHPS subdomains within \textit{Pv}HPPK–DHPS may provide more potent inhibition of the enzyme. This work therefore provides excep-
**Crystal structure of P. vivax HPPK–DHPS enzyme**

Tional opportunities to exploit the structure of PvHPPK–DHPS for screening of drug-like libraries to identify drug scaffolds that can occupy any one or more of its substrate pockets in addition to the pABA-binding site.

**Experimental procedures**

**Gene cloning and protein production**

Full-length *PvHPPK–DHPS* (1–717 amino acids) was purchased as a gBlock (Integrated DNA Technologies, Leuven, Belgium) ORF of full-length *PvHPPK–DHPS* (residues 1–717) was optimized for expression in the *E. coli* strain Rosetta-pLysS and cloned into the pOPINF vector that was linearized using Ncol and Sall restriction sites. Transformed *E. coli* strain Rosetta-pLysS was grown in LB medium containing 100 μg ml⁻¹ ampicillin and 34 μg ml⁻¹ chloramphenicol to an *A*₆₀₀ of 0.6–0.8 at 37 °C. Expression of the His₉-tagged recombinant *PvHPPK–DHPS* was induced by the addition of 0.5 mM isopro- pyl β-D-galactoside, and culture was further incubated at 18 °C for 20 h. Bacterial cells were lysed by a combination of lysozyme treatment and sonication in buffer with 50 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 1 mM phenylmethyl-sulfonyl fluoride, and 1 mM benzamidine HCl. Recombinant protein was affinity captured using nickel–nitrilotriacetic acid–agarose beads (Qiagen) followed by cleavage at 20 °C with 3C-protease for removal of the His₆ tag. Protein was subsequently applied to Q-Sepharose (GE Healthcare) column in buffer with 50 mM Tris, pH 8.0, 250 mM NaCl, 5 mM β-mercaptoethanol. Protein from the flow through fraction was then processed using hydrophobic interaction chromatography on a Phenyl FF 16/10 column (GE Healthcare). Pure fractions obtained from hydrophobic interaction chromatography were pooled and concentrated with 30-kDa cutoff centrifugal devices (Millipore) followed by gel permeation chromatography on a S-200–16/60 column (GE Healthcare) in a buffer containing 50 mM HEPES, pH 6.8, 200 mM NaCl, 5 mM β-mercaptoethanol. A single peak corresponding to dimeric *PvHPPK–DHPS* was collected from gel permeation chromatography.

**Crystallization and data collection**

The purified full-length *PvHPPK–DHPS* (~10 mg ml⁻¹) was used for crystallization screening in the presence of PtPP (0.5 mM), pterin (0.5 mM), AMPCPP (1 mM), and pABA (3 mM) using the hanging-drop vapor-diffusion method at 20 °C with commercially available crystallization screens (Hampton and Molecular Dimensions). The initial screening was carried out in 96-well plates using nano-drop dispensing mosquito robot (TTP Lab Tech). Diffraction quality crystals were obtained using 20% PEG 3350, 0.2 M potassium citrate tribasic monohydrate as mother liquor. The crystals were harvested using corresponding crystallization solution supplemented with 20% (v/v) ethylene glycol as a cryo-protectant and were flash-frozen into liquid nitrogen. Preliminary data collection screening was conducted at PROXIMA 1 Beamline (Soleil, France), and a high-resolution data set was collected at 100 K using Pilatus3 6 m detector (Dectris) and wavelength (λ) of 0.9763 Å at 103 Beamline, Diamond Light Source in the United Kingdom. The data were processed and scaled with XIA2 (39) using DIALS (40), and data processing statistics are shown in Table 1.

**Table 1**

**X-ray data collection and refinement statistics**

The numbers in the parentheses are for the highest resolution bin.

| Source | i03, DLS |
|--------|----------|
| **Data collection** | |
| Wavelength (Å) | 0.9763 |
| Oscillation width (°) | 0.1 |
| Exposure time (s) | 0.02 |
| Cell parameters (Å, °) | 136.05, 113.89, 172.39; 94.24 |
| Space group | P 2₁ |
| Resolution | 113.89–2.79 (2.79–2.84) |
| Number of unique reflections | 129475 (3799) |
| I/σ(I) | 4.5 (0.7) |
| CC₁₆₀ | 0.9 (0.2) |
| Completeness | 99.4 (90.4) |
| Redundancy | 3.5 (3.4) |
| Molecules/ASU | 6 |
| Solvent content (%) | 56 |
| Matthews coefficient Vm (Å³ Da⁻¹) | 2.75 |

| **Refinement** | |
| **Reefinement resolution (Å)** | 50.0–2.90 (2.95–2.90) |
| **Reflections used in refinement** | 114185 |
| **Reflections in working set** | 112179 (8347) |
| **Test set** | 2006 (144) |
| R_{free}/R_{ref} (%) | 24.2/28.5 |

| **Number of atoms** | |
| Protein | 28365 |
| Ligands | 342 |
| Water | 217 |

| **B-factors (Å²)** | |
| Protein | 43.8 |
| Ligands | 46.3 |
| Waters | 18.2 |

| **Ramachandran plot (%)** | |
| Favored regions | 94 |
| Allowed regions | 6 |

| **RMSD** | |
| Bond lengths (Å) | 0.014 |
| Bond angles (°) | 1.643 |

**Structure determination**

Preliminary X-ray data analysis indicated that the *PvHPPK–DHPS* crystals contain ~56% solvent with Matthews coefficient of 2.75 Å³ Da⁻¹ for six full-length *PvHPPK–DHPS* protomers in the ASU. Attempts to solve the phase problem using molecular replacement (MR) techniques with PHASER (41) as implemented in PHENIX (42) and coordinates of fused bifunctional HPPK–DHPS enzyme of *S. cerevisiae* (ScHPPK–DHPS; PDB code 2BMB) and *F. tularensis* (FtHPPK–DHPS; PDB code 4PZV) as template were unsuccessful. In both ScHPPK–DHPS and FtHPPK–DHPS, the orientation of the HPPK domains did not overlap while superposing the DHPS domains. Therefore, the available dimeric DHPS domain structures were fed as template, and most of the MR runs placed three dimers with log-likelihood gain value in the range of 300–600 and with translation function score of 5.5–6.8. The *PvHPPK–DHPS* protomers were manually built, extended and completed by several
cycles of iterative building using COOT (44) and REFMAC (45). Map interpretation and model building was based on electron densities in difference Fourier \( (F_o - F_c) \), \( 2F_o - F_c \) and composite omit maps. In all stages, model building was guided by manual inspection of the model and \( R_{free} \). The substrate/analog and water molecules were added into the difference Fourier maps \( (F_o - F_c) \). The modeled ligands and protein residues were validated using simulated annealing composite omit maps. The occupancies of the ligand molecules were refined and weakly bound ligands, highly disordered loops (residues numbered 1–10, 55–80, 189–202, and 420–434) and low complexity regions (residues 588–660) were not included in the final model. The final refinement statistics are shown in Table 1. The coordinates and structure factors for \( P.v \) HPPK–DHPS have been deposited in the PDB under accession code 5Z79. The figures were generated using CHIMERA (46) and PyMOL (47).

**Author contributions**—M. Y. and Am. S. were responsible for conceptualization, formal analysis, supervision, funding acquisition, and investigation; M. Y. and Am. S. solved the structure of \( P.v \) HPPK–DHPS; J. E. N. and K. H. provided the recombinant clones; K. H. collected diffraction data.; Ak.S. and A.J. performed expression, purification, and crystallization using a construct screened and developed by J. E. N.; R. C., M. S., and J. C. G. maintained the clones; M. Y., Am. S., and J. C. G. analyzed the data and wrote the manuscript.

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