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Mechanistic Binding Insights for 1-Deoxy-D-Xylulose-5-Phosphate Synthase, the Enzyme Catalyzing the First Reaction of Isoprenoid Biosynthesis in the Malaria-Causing Protists, *Plasmodium falciparum* and *Plasmodium vivax*

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Abstract

We have successfully truncated and recombinantly-expressed 1-deoxy-D-xylulose-5-phosphate synthase (DXS) from both *P. vivax* and *P. falciparum*. We elucidated the order of substrate binding for both of these ThDP-dependent enzymes using steady-state kinetic analyses, dead-end inhibition, and intrinsic tryptophan fluorescence titrations. Both enzymes adhere to a random sequential mechanism with respect to binding of both substrates: pyruvate and D-glyceraldehyde-3-phosphate. These findings are in contrast to other ThDP-dependent enzymes, which exhibit classical ordered and/or ping-pong kinetic mechanisms. A better understanding of the kinetic mechanism for these two *Plasmodial* enzymes could aid in the development of novel DXS-specific inhibitors that might prove useful in treatment of malaria.

Keywords

1-deoxy-D-xylulose-5-phosphate synthase; methylerythritol phosphate pathway; isoprenoids; malaria; *Plasmodium vivax*; *Plasmodium falciparum*

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1. Introduction

Malaria is a life-threatening, infectious disease caused by parasites of the genus *Plasmodium*, transmitted through the bite of an infected female *Anopheles* mosquito. Malaria afflicts nearly 100 countries worldwide, with estimates of 198 million cases in 2013, resulting in 584,000 deaths. [1] Malaria poses a risk to roughly half of the population on earth, and infections are on the rise due to global warming, deforestation, and drug and insecticide resistance. [1–5] In humans, malaria is caused by an infection by one of five *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. *P. falciparum* malaria, constituting approximately 75% of the reported cases, is the most deadly form of malaria; however, *P. vivax* malaria, constituting approximately 20% of the reported cases, is serious and more widespread. [1, 6]

Isoprenoids (or terpenoids) constitute one of the largest classes of complex, biologically-active compounds, which serve diverse roles, functioning as cholesterol, hormones, fat-soluble hormones, carotenoids, pigments, and chlorophyll. [7–9] Isoprene is a five-carbon skeleton that is the precursor of all isoprenoids through rearrangement, cyclization, and oxidation. [10] Yet, isoprene itself is not used for the production of isoprenoids *in vivo*; instead the biological forms of isoprene are the two branched, phosphorylated five-carbon precursors, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). For half a century, it was thought that the sole biosynthetic pathway of isoprenoid production was via the mevalonate pathway. [11] However, in 1996, Rohmer *et al.* [12] discovered that certain eubacteria, parasites, and several plants produce isoprenoids from a mevalonate-independent pathway. This biosynthetic route, known as the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, produces DMAPP and IPP from a set of orthogonal enzymes nonexistent in higher organisms and mammals. The MEP pathway, therefore, represents a platform for the development of novel herbicides, antimalarials, and antibiotics to aid the treatment of human disease. [13–16]

In *Plasmodium* parasites, as well as other organisms from the phylum *Apicomplexa*, the MEP pathway produces DMAPP and IPP over seven successive enzymatic reactions. The first committed step of the pathway is catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), which involves the NADPH-dependent rearrangement and two electron reduction of the linear 1-deoxy-D-xylulose-5-phosphate (DXP) to the branched-chain isoprenoid precursor MEP. However, the enzyme catalyzing the first reaction of the pathway is also an attractive target for inhibitor development, 1-deoxy-D-xylulose-5-phosphate synthase (DXS). DXS catalyzes the condensation of pyruvate and D-glyceraldehyde-3-phosphate (GAP) to produce CO₂ and 1-deoxy-D-xylulose-5-phosphate (DXP), a reaction that is the rate-limiting for the MEP pathway in several organisms as well as the branch point for B vitamin and isoprenoid biosynthesis. [17] DXS belongs to the family of enzymes that utilizes thiamine diphosphate (ThDP) as a co-factor, and shares high homology with human transketolase, pyruvate dehydrogenase, and pyruvate decarboxylase. [18–20] Like these other ThDP-dependent enzymes, DXS catalyzes decarboxylation and carbonylation chemistry: a two-carbon intermediate bound to ThDP (hydroxyethyl-ThDP) is condensed with an acceptor substrate (GAP) via nucleophilic addition to generate a new carbon-carbon bond yielding DXP.

All ThDP-dependent enzymes catalyze two successive half reactions. [21] The first step involves the attack of an activated ThDP ylide on the first substrate. [22] The next step could occur via three distinct mechanisms. The most common is the classical ping-pong mechanism, in which the first product is released, forming a metastable enzyme-intermediate complex. Product formation follows after binding of the second substrate. Every ThDP-dependent enzyme studied to date follows this mechanism, and has also been proposed for DXS from *E. coli* and *H. influenza*. [23] Another possibility for the second half reaction proceeds through an ordered mechanism, where the first product can only be released after the second substrate is bound to the active site. The first substrate binds tightly and essentially irreversibly, while the second substrate can only bind to a pocket opened in the presence of the first substrate. Carbon dioxide (CO₂) trapping studies on *R. capsulatus* DXS performed by Eubanks and Poulter suggested an ordered sequential kinetic mechanism. [24] The results of the experiment indicated that pyruvate bound first and irreversibly, followed by binding of GAP to form a ternary complex, and, finally, the release CO₂ and DXP in that order. However, subsequent work demonstrating that DXS will catalyze the conversion of pyruvate to acetolactate and CO₂ at high pyruvate concentrations suggests that the kinetic mechanism for *R. capsulatus* DXS is not sequential. [25, 26]

The third potential mechanism that DXS could display is a random sequential mechanism, in which binding of both substrates is reversible and independent. Brammer *et al.* [25, 27] have proposed an unprecedented rapid equilibrium, random sequential mechanism for *E. coli* DXS, which is in contrast to all known ThDP-dependent enzymes to date. [28] Binding of either pyruvate or GAP is independent of the other, and GAP is required for the release of CO₂ and the formation of a kinetically competent ternary structure. They suggest formation of the lactyl-ThDP intermediate is the overall rate-limiting step and it is maintained in a low-reactive state. GAP binding induces a reorientation to provide the optimal dihedral angle for decarboxylation. [29]

We report, herein, on the successful production of recombinant, catalytically active forms of *Plasmodium falciparum* DXS and *Plasmodium vivax* DXR. Similar to earlier work on the expression of *P. vivax* DXS [30] and *P. falciparum* DXR, [31] the catalytically active forms of *P. falciparum* DXS and *P. vivax* DXR have been truncated to their respective catalytic cores, lacking the signaling and transit peptide for each of these enzymes. In addition to recombinant *P. falciparum* DXS and *P. vivax* DXR, we also produced the truncated forms of *P. vivax* DXS and *P. falciparum* DXR in *E. coli* by following published protocols. Both *P. falciparum* and *P. vivax* DXS enzymes were characterized via steady-state kinetic analyses, intrinsic tryptophan fluorescence titrations, and dead-end inhibition studies employing a pair of pyruvate mimics. DXS activity was measured using a DXR-dependent coupled assay, pairing *P. falciparum* DXS with *P. falciparum* DXR or *P. vivax* DXS with *P. vivax* DXR. Our characterization of DXS from two *Plasmodium* species points toward a random sequential kinetic mechanism, an unusual finding for ThDP-dependent enzymes. This work fosters a deeper understanding of the DXS-catalyzed reaction and will aid in the rational design of DXS inhibitors for the treatment of malaria and other human diseases.

2. Experimental Procedures

2.1. Materials and General Methods

Unless otherwise noted, all reagents were obtained from commercial sources. BL21 (DE3) *E. coli* cells and the *pET-28a* (+) vector were purchased from Novagen. *Pfu*Ultra High-Fidelity DNA polymerase was purchased from Aglient. *Bam*HI, *Xho*I, *Nde*I, Antarctic Phosphatase, and T4 DNA ligase were purchased from New England Biolabs. Kanamycin monosulfate and IPTG were purchased from Gold Biotechnology. Size exclusion chromatography was performed on a GE Healthcare AKTAprime Plus FPLC, coupled to a HiLoad 16/600 Superdex 200 pg column. Spectrophotometric analyses were performed on a Cary 300 Bio UV-Visible spectrophotometer. Tryptophan fluorescence studies were performed on a JASCO FP-8300 spectrofluorometer.

2.2. Cloning of the *P. falciparum* dxs and *P. vivax* dxr Genes Without Signal and Transit Peptides

A synthetic, codon-optimized gene for *P. falciparum* dxs was purchased from Genscript. *P. falciparum* dxs was designed with 5'-*Nde*I and 3'-*Xho*I restriction sites and synthesized into a *pUC57* vector. The full-length gene was excised from the *pUC57* vector and cloned into the *Nde*I and *Xho*I restriction sites of the *pET-28a* vector. Similarly, a *P. vivax* dxr codon-optimized gene was also purchased from Genscript, with 5'-*Nde*I and 3'-*Bam*HI restriction sites in a *pUC57* vector. *P. vivax* dxr was cloned into *pET-28a* using *Nde*I and *Bam*HI restriction sites.

Truncated gene constructs were produced from the full length, codon-optimized genes using PCR extraction to eliminate expression of the N-terminal signal and transit peptides of both *Pf*DXS and *Pv*DXR. *Pf*DXS-A, *Pf*DXS-B, *Pf*DXS-C, and *Pf*DXS-D were produced with deletions of 237, 286, 292, and 310 amino acids, respectively. *Pv*DXR-E, *Pv*DXR-F, and *Pv*DXR-G were constructed with 99, 106, and 110 amino acid deletions, respectively. The cloning of *Pv*DXS was described previously. [30] The cloning of *Pf*DXR has also been previously described, [31] and this gene was generously donated by Dr. Nobutada Tanaka at Showa University (Japan) for the use in this study.

2.3. Overexpression of Truncated DXS and DXR Proteins in *E. coli*

Truncated gene transcripts of DXS and DXR in *pET-28a* were transformed into *E. coli* BL-21 (DE3) cells. A fresh colony of cells harboring either truncated *Pf*dxs-*pET-28a* or *Pv*dxr-*pET-28a* were inoculated at 37 °C in LB broth containing 40 µg/mL kanamycin supplemented with 0.8% glucose and 25 mM potassium phosphate (pH = 7.2). The cultures were grown until the OD₆₀₀ reached 0.3, then diluted 1:100. For the overexpression of *Pf*DXS, cells were cultured until the OD₆₀₀ reached 0.6, the temperature reduced to 10 °C, induced by the addition of 0.5 mM IPTG, and incubated overnight (12 hours) with continuous shaking. To produce *Pv*DXR, the temperature was dropped to 30 °C after achieving an OD₆₀₀ of 0.6, induction initiated with 1 mM IPTG, and overnight incubation. Cells were harvested by centrifugation, and the cell pellets stored at -80 °C. The expression of *Pv*DXS [30] and *Pf*DXR [31] were described previously.

2.4. Purification of DXS and DXR by Ni-NTA Affinity and Size-Exclusion Chromatography

Cells were thawed on ice and all purification steps were performed at 4 °C. Cell pellets (4 g) were resuspended in 20 mL of binding buffer supplemented with protease inhibitors: 20 mM Tris pH 7.5, 500 mM NaCl, 20 mM imidazole, 10 mM b-mercaptoethanol (BME), 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 mg/mL leupeptin, 2 mg/mL pepstatin, and 43 mg of an *E. coli*-specific protease inhibitor cocktail (Sigma P8465). The cells were lysed via sonication, centrifuged at $16,000 \times g$ for 20 minutes at 4 °C, and the recombinant enzyme-containing soluble lysate was retained.

Purification of DXS and DXR was facilitated by the fusion of an N-terminal His₆-tag to the active, truncated enzymes. The supernatant was applied to a borosilicate glass column (1.5 cm $\times$ 5 cm) containing 5 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated with binding buffer. The non-bound proteins were first eluted with 5 column volumes (CVs) of binding buffer, then with 20 CVs of wash buffer (20 mM Tris pH 7.5, 500 mM NaCl, 60 mM imidazole, and 10 mM BME) at a flow rate of 1 mL/minute. Tagged, recombinant enzyme was eluted with 2 CVs of elution buffer (20 mM Tris pH 7.5, 500 mM NaCl, 250 mM imidazole, 10 mM BME) and concentrated to <3 mL using an Amicon stirred ultrafiltration cell in preparation for size exclusion chromatography (SEC). The concentrated sample was injected onto an AKTApurify FPLC coupled to a 16/600 Superdex column equilibrated with 50 mM Tris pH 7.5, 150 mM NaCl, and 10 mM BME. The flow rate for the SEC was set to 1 mL/min with a maximum pressure of 0.5 mPa. The concentration of the enzyme-containing fractions was determined using the Bradford assay and purity was assessed by a 10% SDS-PAGE gel visualized with Coomassie Brilliant Blue stain. Fractions containing purified enzyme were combined, glycerol added to a final concentration of 10% (w/v), and the aliquots were flash frozen in liquid nitrogen with subsequent storage at -80 °C.

2.5. Kinetic Analyses

DXS activity from both *P. falciparum* and *P. vivax* was measured spectrophotometrically using DXR from its corresponding organism as a coupling enzyme as previously reported [32] with minor variations. The concentration of D-GAP was determined enzymatically from commercially available D,L-GAP using human GAPDH. [26] Throughout the manuscript, our use of “GAP” refers to the concentration of D-GAP in the D,L-GAP mixture. Reaction mixtures containing 100 mM HEPES pH 7.0, 100 mM NaCl, 1.5 mM MgCl₂, 1 mg/mL BSA, 1 mM ThDP, 2 mM BME, 150 μ M NADPH, GAP, and pyruvate were preincubated for 5 minutes at 37 °C. DXR was pipetted into the cuvette (0.2 mg/mL), 100 nM of DXS was added to initiate the reaction, and the solution (750 μ L total volume) was vigorously mixed before initializing the spectrophotometer software. Rates of NADPH consumption in the coupled step between DXS and DXR were monitored spectrophotometrically at 340 nm for 2 minutes (extinction coefficient of $6,220 \text{ M}^{-1} \text{ cm}^{-1}$ for NADPH). The rates of NADPH depletion were used to calculate initial rates of DXP formation. The apparent kinetic constants were determined by fitting the resulting data to equation 1 using SigmaPlot 12.0: v_0 represents initial velocity, $V_{m,app}$ is the apparent maximal velocity, $K_{m,app}$ is the apparent Michaelis constant, and $[S]$ is the substrate

concentration. Assays were performed in triplicate and the uncertainty for the $k_{\text{cat,app}}$ and $(k_{\text{cat}}/K_m)_{\text{app}}$ values were calculated using equation 2, where σ is the standard error. [33]

$$v_o = \frac{V_{m,\text{app}}[S]}{K_{m,\text{app}} + [S]} \quad \text{Equation 1}$$

$$\sigma\left(\frac{x}{y}\right) = \frac{x}{y} \sqrt{\left(\frac{\sigma_x}{x}\right)^2 + \left(\frac{\sigma_y}{y}\right)^2} \quad \text{Equation 2}$$

To delineate between a sequential and classic ping-pong mechanism, double-reciprocal plots of the initial velocity data were generated for GAP and pyruvate in SigmaPlot 12.0. Non-linear regression analysis of initial rates and model discrimination analyses were performed in WaveMetrics IGOR Pro 6. Initial velocities were determined by varying the concentration of one substrate, while holding the other substrate at a fixed concentration. GAP was evaluated between 3.5 μM and 16 μM , whereas pyruvate was tested at concentrations ranging from 50 μM to 250 μM . The resulting initial velocity data was fit to equation 3 for a rapid-equilibrium random Bi-Bi mechanism, where v_o is the initial velocity, V_m is the maximal velocity, $[A]$ is the concentration of substrate A, $[B]$ is the concentration of substrate B, K_{ia} is the dissociation constant for substrate A, K_b is the Michaelis constant for substrate B, and K_a is the Michaelis constant for substrate A.

$$v_o = \frac{V_{\text{max}}[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]} \quad \text{Equation 3}$$

2.6. Synthesis of Methylacetylphosphonate (MAP)

MAP was synthesized, as previously reported [25, 34–36], with minor variations. In a dry three-necked round bottom flask with a magnetic stir bar under positive N_2 pressure, acetyl chloride (1.21 mL, 14 mmol, 1 eq) was cooled to 0 $^{\circ}\text{C}$ in an ice-water bath. Trimethyl phosphite (1.65 mL, 14 mmol, 1 eq) was slowly injected over a period of 3 hours, which resulted in evolution of MeCl. After full addition of trimethyl phosphite, the light-grey solution was allowed to warm to room temperature and stirred for an additional 8 hours. Unreacted reagents were removed under vacuum to afford acetylphosphonate dimethyl ester (DAP). 1.58 g recovered (10.4 mmol, 74% yield) as a pale grey oil. ^1H NMR (400 MHz, CDCl_3) δ 2.24 (d, $J = 5.31$ Hz, 3H, CH_3), 3.61 (d, $J = 10.69$, 6H, OCH_3). ^{13}C NMR (400 MHz, CDCl_3): δ 208.1 (d, $\text{JP}, \text{C} = 169.8$ Hz), 54.0 (d, $2\text{JP}, \text{C} = 7.2$ Hz, 2 C), 20.9 (d, $2\text{JP}, \text{C} = 36.0$ Hz).

The recovered DAP (1.58 g, 10.4 mmol, 1 eq) was dissolved in dry acetone (10.4 mL), and the solution was slowly injected into a solution of sodium iodide (1.71 g, 11.5 mmol, 1.1 eq) in 6 mL dry acetone. The yellow reaction mixture was stirred overnight at room temperature after which the precipitate was filtered, triturated with dry acetone, and dried to yield purified MAP. 93% yield. ^1H NMR (400 MHz, D_2O) δ 2.20 (3 H, d, $J = 9.8$ Hz) and 3.69 (3 H, d, $J = 10.63$ Hz). All NMR spectra of isolated compounds are in agreement with literature reports.

2.7. Inhibition of DXS with β -Fluorpyruvate and Methylacetylphosphonate

The varying inhibition patterns of DXS activity were measured with the DXS-DXR coupled assay in the presence of either β -fluorpyruvate or methylacetylphosphonate (MAP). Inhibitor concentrations were based upon previously established IC_{50} values (data not shown) and added to the kinetic reaction mixtures described above. For *Pv*DXS, the concentrations of β -fluorpyruvate tested were 0, 35, and 75 μ M, while MAP was analyzed at 0, 150, and 300 μ M. To test *Pf*DXS inhibition, β -fluorpyruvate and MAP concentrations were held at 0, 50, 100 μ M and 0, 70, 140 μ M, respectively. No inhibition of DXR by either inhibitor was observed. All assays were performed in triplicate, and the resulting data was fit to equations 4 – 6 in SigmaPlot12.0 for competitive, noncompetitive, and uncompetitive inhibition, respectively. For equations 4 – 6, v_o is the initial velocity, $V_{m,app}$ is the apparent maximal velocity, $K_{m,app}$ is the apparent Michaelis constant, $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration, and K_i is the inhibition constant.

$$v_o = \frac{V_{m,app}[S]}{K_{m,app} \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad \text{Equation 4}$$

$$v_o = \frac{V_{m,app}[S]}{K_{m,app} \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{K_i}\right)} \quad \text{Equation 5}$$

$$v_o = \frac{V_{m,app}[S]}{K_{m,app} + [S] \left(1 + \frac{[I]}{K_i}\right)} \quad \text{Equation 6}$$

2.8. Intrinsic Tryptophan Fluorescence Measurements for the Determination of Binding Constants for DXS

To study the binding of pyruvate and GAP to DXS, intrinsic tryptophan fluorescence (ITF) quenching titrations were utilized. All reaction mixtures for ITF titrations contained 100 mM HEPES pH 7.0, 100 mM NaCl, 1.5 mM $MgCl_2$, 2 mM BME, and 500 nM DXS in a final volume of 400 μ L. Fluorescent emission spectra were acquired on a JASCO FP-8300 spectrofluorometer at 25 °C. The excitation wavelength was set at 280 nm, and emission occurred between 290–300 nm. Spectra were measured in triplicate from 305 nm to 360 nm, using a scan speed of 50 nm/min, and both excitation and emission bandwidth set at 2.5 nm. Spectra were corrected by subtracting emission spectra from acquisitions containing no enzyme (blanks), and normalized by taking the ratio of the fluorescence emission intensity at the λ_{max} relative to starting enzyme only samples. Data were fit to equation 7, where ΔF is the change in intrinsic fluorescence, F_{max} is the maximum change of fluorescence at infinite ligand concentration, $[L]$ is the ligand concentration, and K_d is the ligand dissociation constant.

$$\Delta F = \frac{F_{max}[L]}{K_d + [L]} \quad \text{Equation 7}$$

3. Results and Discussion

Malaria parasites and other infectious organisms of the phylum *Apicomplexa* possess the apicoplast, an endosymbiotic organelle similar to the chloroplast of plants and algae. [37] With a complete genome producing nearly 400 assorted proteins, the apicoplast is vital in various aspects of parasite metabolism including fatty acid and isoprenoid biosynthesis. All of the enzymes from the MEP pathway are nuclear-encoded and translocated to the apicoplast through a secretory pathway utilizing a bipartite N-terminal signaling motif. This sequence consists of two functional domains: an endoplasmic reticulum-targeting signal peptide and a transit peptide. The signal peptide directs the translocation of the enzyme into the secretory pathway, while the transit peptide facilitates the movement through the four outer membranes of the apicoplast. Once inside, the peptides are cleaved off (most likely by a stromal processing protease) to yield the catalytically-competent enzyme. [38]

To date, only a few enzymes targeted to the apicoplast from *Plasmodium* have been expressed, due in part to difficulties in obtaining soluble recombinant enzyme from *E. coli* host cells. [30, 39, 40] In addition to codon biases between *E. coli* and *Plasmodium* species (high GC content, ribosomal binding sites, random repeats of rare codons, etc), the presence of the signaling and transit motifs consistently result in the accumulation of insoluble inclusion bodies. [39] Therefore, serial deletion of the full-length, untruncated gene is required to produce a DXS or DXR variant that is soluble when expressed in *E. coli*. Several freely available algorithms (PlasmoAP [41], PATS [42], PSORT [43], etc.) have been developed, which have proven successful in identifying the signal peptide. Note that the signal peptide varies in length from 20–30 amino acids and exhibits a consensus sequence. In contrast, there is no consensus sequence or narrowly defined size for the transit peptide, which can vary from 50–500 amino acids, meaning that there are no current algorithms that can accurately predict this peptide region (see Figure 1 of the DXS amino acid sequence from several organisms with low N-terminal homology). [20] Malarial transit peptides can be approximated by the presence of highly enriched lysine and asparagine residues, which results in an overall positive charge. [41] This trend is pronounced in the amino acid sequence of *P. falciparum* DXS between residues ~150–300 (Figure 1, note the high number of successive asparagine residues).

By utilizing homology modeling and N-terminal serial deletions of the full-length codon-optimized genes, several targeted variants of *PfDXS* and *PvDXR* were produced in our expression optimization study. *PfDXS*-A, *PfDXS*-B, *PfDXS*-C, and *PfDXS*-D were generated with N-terminal deletions of 237, 286, 292, and 310 amino acids, respectively. *PvDXR*-E, *PvDXR*-F, and *PvDXR*-G were constructed with N-terminal deletions of 99, 106, and 110 amino acid deletions, respectively. Each of these genes was then transformed into BL-21 (DE3) *E. coli* expression cells, and cultured in 2 mL aliquots. These small-scale cultures were optimized for time of expression (1, 3, 5, and 12 hours), induction temperature (10, 25, 30, or 37 °C) and IPTG concentration (0.1, 0.5, or 1.0 mM) for a total of 48 culture conditions per truncated gene. The small scale cultures were then centrifuged, lysed via sonication, and the soluble proteome subjected to an anti-His₆ Western blot antibody assay for the detection of recombinant enzyme. Only one truncated gene and one set of variables proved successful for each enzyme: *PfDXS*-D (with 310 amino acids deleted) was expressed

at 10 °C, induced by the addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and incubated overnight (12 hours) with continuous shaking. After subsequent purification via Ni-NTA affinity and size exclusion chromatography, the final yield of *Pf*DXS-D was 1.4 mg/L (Figure 2). To produce *Pv*DXR, the protein construct with 110 amino acids removed (*Pv*DXR-G) produced soluble enzyme at 30 °C with 1 mM IPTG and overnight incubation. *Pv*DXR-G yielded 0.9 mg/L after both affinity and size exclusion chromatography (Figure 3). This enzyme was used in the DXS-DXR coupled assay to further study *Pv*DXS (kinetic constants of *Pv*DXR-G: $K_m = 48 \pm 1.6 \mu\text{M}$, $V_m = 1.3 \pm 0.01 \text{ s}^{-1}$, $k_{\text{cat}}/K_m = (2.7 \pm 0.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$). These values are in good agreement with previously published data for DXR from several other organisms. [44–47] The cloning, expression, and characterization of *Pf*DXR [31] and *Pv*DXS [30] were previously described. From this point forth, *Pf*DXS-D and *Pv*DXR-G will simply be referred to as *Pf*DXS and *Pv*DXR, respectively.

Once both sets of enzymes were cloned, expressed, and purified, we set about to elucidate kinetic and binding characteristics of DXS from *P. falciparum* and *P. vivax*. For the determination of kinetic constants, initial velocities of DXP formation were monitored using the DXS-DXR coupled assay at sub-saturating concentrations of one substrate, while holding the other fixed. Rates were determined in triplicate. Binding constants were obtained using intrinsic tryptophan fluorescence titrations, and the results of both of these experiments are detailed in Table 1.

The kinetic constants for both *P. falciparum* DXS and *P. vivax* DXS are comparable to previously published data for DXS from *E. coli*, *R. capsulatus*, and *D. radiodurans*. The K_d values for both *Plasmodium* enzymes are also commensurate with the dissociation constants for *E. coli* DXS (see Figures 4 and 5). Clues of the binding mechanism can be delineated from these data: in the ordered mechanism with pyruvate binding first and, irreversibly, binding of pyruvate causes a conformational change which promotes binding of GAP. Binding of GAP to free enzyme should, therefore, be negligible. [25] However, a measurable binding constant for pyruvate to both *Pf*DXS and *Pv*DXS suggests that pyruvate binding is, in fact reversible, inconsistent with an ordered mechanism. In the proposed ping-pong mechanism, GAP will have low affinity for the free enzyme. However, again, we observed quenching of the fluorescent signal indicative of GAP binding reversibly to both of the *Plasmodium* DXS enzymes. These data are inconsistent with a ping-pong mechanism. Taken together, the intrinsic fluorescence data supports a random sequential mechanism where GAP and pyruvate can both bind reversibly and independently to either *P. falciparum* or *P. vivax* DXS.

Also noted in our ITF data is an apparent Stokes shift in the wavelength for maximum fluorescence intensity when DXS is incubated with high concentrations of GAP, which was *not* found at similarly high levels of pyruvate (Figure 4). Tryptophan fluorescence is commonly used to monitor perturbations in protein dynamics and localized structures; fluorescence from tryptophan is sensitive to the polarity of its local environment. [48] Therefore, this apparent Stokes shift suggests that GAP binding induces a conformational change in DXS, at least within the vicinity of the fluorescent tryptophan residue. This represents the first report of a conformational change in DXS upon substrate binding.

Additional data for other DXS enzymes is required to determine the significance of our observation.

Double-reciprocal plots were generated to differentiate between a sequential binding mechanism and a ping-pong mechanism for both of the DXS enzymes from *Plasmodium* (Figures 6 and 7). The Lineweaver-Burk plots for both GAP and pyruvate had noncompetitive pattern with changes in both slope and intercept, suggesting a random sequential mechanism. If substrate binding to DXS were ordered, a competitive pattern for GAP would result and an uncompetitive pattern for pyruvate would be observed. Substrate inhibition was found at higher concentrations of both pyruvate and GAP (as evidenced by the converging lines), similar to what was reported by Brammer, *et al.* [25] for *E. coli* DXS. The data was further evaluated using global non-linear regression analysis: several mechanistic models for a two substrate, two product (Bi Bi) system were examined in a model discrimination analysis (rapid-equilibrium ordered, rapid-equilibrium random, ping-pong, etc). The data were best fit to the equations for a random sequential mechanism (Figures 8 and 9).

Finally, inhibition studies were conducted to further confirm the mechanism of substrate binding to DXS. Both β -fluoropyruvate and MAP have been previously used as pyruvate mimics to study ThDP-dependent enzyme mechanisms. [34, 49–51] After ThDP activation, β -fluoropyruvate undergoes a catalytic step to release CO₂ with consequent elimination of fluoride; therefore, it is not representative of a true dead-end inhibitor against pyruvate. MAP cannot undergo decarboxylation, and is, accordingly, a more suitable pyruvate analog. [25] Initial rates of DXP production were monitored using the DXS-DXR coupled assay at sub-saturating, varied concentrations of one substrate at fixed concentrations of the second substrate, in the presence of either inhibitor at varied concentrations. The equations for competitive, noncompetitive, and uncompetitive inhibition were fit to the data, and the results of the best fits are shown in Figures 10 and 11. For the pair of DXS enzymes, both MAP and β -fluoropyruvate displayed competitive inhibition against pyruvate, while exhibiting noncompetitive inhibition versus GAP (Table 2). This pattern of results is most consistent with a random sequential binding mechanism for both substrates to DXS. Conversely, a ping-pong mechanism would yield an uncompetitive pattern with respect to GAP.

4. Conclusion

The first two enzymes of the MEP pathway in the malaria-causing protists *Plasmodium vivax* and *Plasmodium falciparum* are DXS and DXR, respectively. Catalytically active, truncated forms of all four enzymes have been cloned and over-expressed in *E. coli*: *P. falciparum* DXS (described herein), *P. falciparum* DXR by Umeda *et al.*²⁶, *P. vivax* DXS by Handa *et al.*²⁵, and *P. vivax* DXR (described herein). Because of the metabolic centrality of the isoprenoids, the availability of DXS and DXR from *P. falciparum* and *P. vivax* should facilitate the development of novel anti-malarials targeted against either of these enzymes. The kinetic mechanism for DXS has proven controversial, with the majority of the published data pointing towards a random sequential mechanism. [25, 27, 28] Such a mechanism is unusual for a ThDP-dependent enzyme, as the first substrate typically forms an adduct with

the thiazolium moiety of ThDP. [21, 22, 27] yielding either an ordered or ping-pong kinetic mechanism. Our characterization of *Pf*DXS and *Pv*DXS, steady-state kinetic analyses, substrate binding studies, and dead-end inhibition experiments, are most consistent with a random sequential mechanism for both enzymes. The expression of *Pf*DXS and *Pv*DXS in *E. coli*, providing straightforward access to multi-milligram amounts of protein and fostering the construction of site-directed mutants, will enable the detailed structure-function studies to better understand the unique aspects of ThDP-dependent chemistry of DXS.

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Abbreviations

DXS	1-deoxy-D-xylulose-5-phosphate synthase
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
IPP	isopentyl diphosphate
IPTG	isopropyl β -D-1-thiogalactopyranoside
DMAPP	dimethylallyl diphosphate
MEP	2-C-methyl-D-erythritol 4-phosphate
GAP	D-glyceraldehyde-3-phosphate
DXP	1-deoxy-D-xylulose-5-phosphate
ThDP	thiamine diphosphate
MAP	methylacetylphosphonate

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Highlights

- The novel truncation and expression of *Plasmodium falciparum* DXS and *Plasmodium vivax* DXR.
- Enzymatic characterization by steady-state kinetics, inhibition, and binding constants.
- Confirmed previous reports of a random sequential binding mechanism for both *Plasmodium falciparum* and *Plasmodium vivax* DXS.

Figure 1.

Homology overlays of the N-terminal amino acid sequence for *Plasmodium vivax*, *Plasmodium knowlesi*, *Plasmodium cynomolgi* and *Plasmodium falciparum*.

Figure 2. Purification scheme of *Plasmodium falciparum* DXS.

(A) Size-exclusion chromatography elution profile versus enzymatic activity.

(B) SDS-PAGE of the purification workflow

1. Molecular weight ladder
2. Nickel-column binding fraction
3. Nickel-column wash fraction
4. Nickel-column elution fraction
5. Size-exclusion column load
6. Size-exclusion column -purified *PfDXS*
7. MagicMark XP Western blot molecular weight ladder

(C) Western blot of the purification workflow

1. Molecular weight ladder
2. Nickel-column binding fraction
3. Nickel-column wash fraction
4. Nickel-column elution fraction
5. Size-exclusion column load
6. Size-exclusion column -purified *PfDXS*
7. MagicMark XP Western blot molecular weight ladder

Figure 3.
Purification scheme of *Plasmodium vivax* DXR.

(A) Same as A above

(B) Same as B above

(C) Same as C above

Figure 4. Substrate-binding analysis of *PvDXS* by intrinsic-tryptophan quenching titrations with increasing [pyruvate] and [GAP].

Figure 5. Substrate-binding analysis of *PfDXS* by intrinsic-tryptophan quenching titrations with increasing [pyruvate] and [GAP].

Figure 6.

PvDxs double-reciprocal plots of initial velocities for pyruvate and GAP. (A) Velocities measured at fixed concentrations of GAP: 0.016 mM (★), 0.008 mM (●), 0.005 mM (■), 0.0035 mM (▲). (B) Velocities measured at fixed concentrations of pyruvate: 0.25 mM (★), 0.15 mM (●), 0.10 mM (■), and 0.05 mM (▲).

Figure 7.

PjDXS double-reciprocal plots of initial velocities for pyruvate and GAP. (A) Velocities measured at fixed concentrations of GAP: 0.016 mM (★), 0.008 mM (●), 0.005 mM (■), 0.0035 mM (▲). (B) Velocities measured at fixed concentrations of pyruvate: 0.25 mM (★), 0.15 mM (●), 0.10 mM (■), and 0.05 mM (▲).

Figure 8. *PvDXS* global non-linear regression analysis for a random sequential mechanism. (A) Velocities measured at fixed concentrations of GAP: 0.016 mM (Δ), 0.008 mM ($\square$), 0.005 mM ($\diamond$), 0.0035 mM ($\circ$). (B) Velocities measured at fixed concentrations of pyruvate: 0.25 mM (Δ), 0.15 mM ($\square$), 0.10 mM ($\diamond$), and 0.05 mM ($\circ$).

Figure 9.

PfDXS global non-linear regression analysis for a random sequential mechanism. (A)

Velocities measured at fixed concentrations of GAP: 0.016 mM (Δ), 0.008 mM ($\square$), 0.005

mM ($\circ$), 0.0035 mM ($\diamond$). (B) Velocities measured at fixed concentrations of pyruvate: 0.25

mM (Δ), 0.15 mM ($\square$), 0.10 mM ($\circ$), and 0.05 mM ($\diamond$).

Figure 10.

Inhibition of DXS by β -fluoropyruvate. (A) *Pv*DXS assayed with varied concentrations of pyruvate at fixed concentrations of GAP (26 μM), in the presence of β -fluoropyruvate: 0 μM (●), 35 μM (○), and 75 μM (▼). (B) *Pv*DXS assayed with varied concentrations of GAP at fixed concentrations of pyruvate (1.1 mM), in the presence of β -fluoropyruvate: 0 μM (●), 35 μM (○), and 75 μM (▼). (C) *Pf*DXS assayed with varied concentrations of pyruvate at fixed concentrations of GAP (16 μM), in the presence of β -fluoropyruvate: 0 μM (●), 50 μM (○), and 100 μM (▼). (D) *Pf*DXS assayed with varied concentrations of GAP at fixed concentrations of pyruvate (640 μM), in the presence of β -fluoropyruvate: 0 μM (●), 50 μM (○), and 100 μM (▼).

Figure 11.

Inhibition of DXS by MAP. (A) *Pv*DXS assayed with varied concentrations of pyruvate at fixed concentrations of GAP (26 μM), in the presence of MAP: 0 μM (●), 150 μM (○), and 300 μM (▼). (B) *Pv*DXS assayed with varied concentrations of GAP at fixed concentrations of pyruvate (1.1 mM), in the presence of MAP: 0 μM (●), 150 μM (○), and 300 μM (▼). (C) *Pf*DXS assayed with varied concentrations of pyruvate at fixed concentrations of GAP (16 μM), in the presence of MAP: 0 μM (●), 70 μM (○), and 140 μM (▼). (D) *Pf*DXS assayed with varied concentrations of GAP at fixed concentrations of pyruvate (640 μM), in the presence of MAP: 0 μM (●), 70 μM (○), and 140 μM (▼).

Table 1

Binding Constants and Steady-State Kinetic Constants of DXS from several organisms

Organism	Pyruvate GAP			GAP					
	K_m, app (μM)	K_d (μM)	$k_{cat, app}$ (s^{-1})	$(k_{cat}/K_m)_{app}$ ($M^{-1} s^{-1}$)	K_m, app (μM)	K_d (μM)	$k_{cat, app}$ (s^{-1})	$(k_{cat}/K_m)_{app}$ ($M^{-1} s^{-1}$)	Reference
<i>P. falciparum</i>	640 $\pm$ 16	2600 $\pm$ 650	4.2 $\pm$ 0.03	(6.5 $\pm$ 0.2) $\times 10^3$	16 $\pm$ 2.3	4100 $\pm$ 1200	4.1 $\pm$ 0.17	(2.5 $\pm$ 0.4) $\times 10^5$	1
<i>P. vivax</i>	1090 $\pm$ 60	770 $\pm$ 210	6.6 $\pm$ 0.15	(6.1 $\pm$ 0.4) $\times 10^3$	26 $\pm$ 2.6	2300 $\pm$ 1300	6.4 $\pm$ 0.22	(2.4 $\pm$ 0.3) $\times 10^5$	1
<i>E. coli</i>	49 $\pm$ 8.0	230 $\pm$ 70	2.6 $\pm$ 0.11	5.3 $\times 10^4$	24 $\pm$ 1.7	2000 $\pm$ 500	2.6 $\pm$ 0.11	1.1 $\times 10^5$	2
<i>R. capsulatus</i>	440 $\pm$ 50	--	1.9 $\pm$ 0.10	4.3 $\times 10^3$	68 $\pm$ 1.0	--	1.9 $\pm$ 0.10	2.8 $\times 10^4$	3
<i>D. radiodurans</i>	280 $\pm$ 30	--	7.4 $\pm$ 0.30	2.6 $\times 10^4$	50 $\pm$ 10.0	--	7.9 $\pm$ 0.40	1.5 $\times 10^5$	4

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Table 2Inhibition constants for β -fluoropyruvate and MAP against *Pv*DXS and *Pf*DXS

Enzyme	Inhibitor	Substrate	Pattern	$K_{i\text{ app}}$ (μM)
<i>Pv</i> DXS	β -fluoropyruvate	Pyruvate	Competitive	35 ± 1.7
		GAP	Noncompetitive	77 ± 7.2
	MAP	Pyruvate	Competitive	75 ± 5.0
		GAP	Noncompetitive	89 ± 6.7
<i>Pf</i> DXS	β -fluoropyruvate	Pyruvate	Competitive	43 ± 3.8
		GAP	Noncompetitive	131 ± 10
	MAP	Pyruvate	Competitive	46 ± 3.8
		GAP	Noncompetitive	103 ± 7.2