1-Deoxy-D-xylulose 5-Phosphate Synthase Catalyzes a Novel Random Sequential Mechanism

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Background: 1-Deoxy-D-xylulose 5-phosphate (DXP) synthase is a thiamine diphosphate (ThDP)-dependent enzyme in pathogen isoprenoid biosynthesis and a potential drug target.

Results: Tryptophan fluorescence and kinetic analyses show that donor and acceptor substrates bind reversibly and independently to DXP synthase.

Conclusion: DXP synthase catalyzes a novel, ThDP-dependent, random sequential mechanism.

Significance: Targeting the unique kinetic mechanism of DXP synthase could lead to new anti-infective agents.

Emerging resistance of human pathogens to anti-infective agents make it necessary to develop new agents to treat infection. The methylerythritol phosphate pathway has been identified as an anti-infective target, as this essential isoprenoid biosynthesis and a potential drug target.

The first committed step in non-mammalian isoprenoid biosynthesis is the production of methylerythritol phosphate by the action of reductoisomerase IspC (Fig. 1). The natural substrate for this reaction, 1-deoxy-D-xylulose 5-phosphate (DXP),4 is generated from pyruvate and D-glyceraldehyde 3-phosphate (D-GAP) in a thiamine diphosphate (ThDP)-dependent manner. Structural analysis has revealed a unique domain arrangement suggesting opportunities for the selective targeting of DXP synthase; however, reports on the kinetic mechanism are conflicting. Here, we present the results of tryptophan fluorescence binding and kinetic analyses of DXP synthase and propose a new model for substrate binding and mechanism. Our results are consistent with a random sequential kinetic mechanism, which is unprecedented in this enzyme class.

Members of the isoprenoid natural product class are constructed from the precursors isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which are essential in all living organisms. For decades, the mevalonate pathway was believed to be the universal source of isopentenyl pyrophosphate and dimethylallyl pyrophosphate. It is now known that the mevalonate pathway is utilized by eukaryotes, archa, and some plant species, whereas the recently elucidated methylerythritol phosphate pathway (Fig. 1) (1) is the source of isopentenyl pyrophosphate and dimethylallyl pyrophosphate in many green algae, plants, eubacteria, and apicomplexan protozoa (2). The methylerythritol phosphate pathway enzymes are widespread in human pathogens and are therefore being pursued as potential drug targets toward the development of broad spectrum anti-infective agents (3, 4).

The first committed step in non-mammalian isoprenoid biosynthesis is the production of methylerythritol phosphate by the action of reductoisomerase IspC (Fig. 1). The natural substrate for this reaction, 1-deoxy-D-xylulose 5-phosphate (DXP),4 is generated from pyruvate and D-glyceraldehyde 3-phosphate (D-GAP) in a thiamine diphosphate (ThDP)-dependent reaction catalyzed by DXP synthase (5). This first step is believed to be rate-limiting in some organisms (6, 7) and represents a branch point in bacterial metabolism (5, 8, 9). The essential role of DXP synthase in pathogen metabolism, as well as its putative regulatory role in isoprenoid biosynthesis, point to this enzyme as a potential target for anti-infective drug development.

The reaction catalyzed by DXP synthase combines elements of ThDP-dependent decarboxylase and carboligase chemistry (5), and active site residues necessary for coordinating the cofactor are conserved relative to other ThDP-dependent enzymes (10). DXP synthase catalyzes the decarboxylation of pyruvate and subsequent condensation of the resulting hydroxymethyl-ThDP intermediate with D-GAP. This reaction is reminiscent of acetolactate synthase and glyoxylate carboligase, which generate acetohydroxyacid and tartronate semialdehyde, respectively, via similar decarboxylation and carboligation chemistry, as well as transketolase whose acceptor substrate is also an aldehyde. Interestingly, DXP synthase shows some unique structural features relative to other ThDP-dependent enzymes (10). The enzyme is homodimeric, possessing a distinct domain arrangement with the active site positioned between domains of the same monomer. This is in contrast to other structurally similar ThDP-dependent enzymes, including transketolase and pyruvate dehydrogenase E1 subunit, where the active site is positioned at the dimer interface (10, 11).

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4 The abbreviations used are: DXP, 1-deoxy-D-xylulose 5-phosphosphate; D-GAP, D-glyceraldehyde 3-phosphate; ThDP, thiamine diphosphate; MAP, methylacetylphosphonate.
Early work on the kinetic mechanism of *Rhodobacter capsulatus* DXP synthase suggests this enzyme is also mechanistically distinct from other ThDP-dependent enzymes (12) despite the similar carboligation transformation it catalyzes. All other ThDP-dependent enzymes are believed to operate via a classical ping-pong mechanism (11), whereas CO₂ trapping studies performed by Eubanks and Poulter (12) have provided compelling evidence for the requirement of a ternary complex in DXP synthase catalysis. Interestingly, slow CO₂ release was observed in the absence of D-GAP, which can be explained by the aceto-lactate synthase activity as a side reaction of DXP synthase reported by our group in a subsequent study of DXP synthase substrate specificity (13). In addition, an ordered substrate binding model was proposed (12), in which pyruvate binds essentially irreversibly to the free enzyme prior to binding of D-GAP (Fig. 2A). A more recent study reported a single-molecule force spectroscopy nanosensor to measure *E. coli* DXP synthase substrate binding and application of single-molecule force spectroscopy for identifying /H9252-fluoropyruvate as an inhibitor of the enzyme (14). Using immobilized enzyme and substrate, the authors determined substrate binding constants and suggested that the observed 1.7-fold enhancement in binding of D-GAP to DXP synthase in the presence of soluble pyruvate provides further support for an ordered mechanism. A conflicting report (15) proposes, on the basis of steady-state kinetics, that *Escherichia coli* and *Haemophilus influenzae* DXP synthase operate via a classical ping-pong mechanism in a manner similar to other ThDP-dependent enzymes (Fig. 2B).

Our interest in pursuing the development of selective DXP synthase inhibitors requires detailed knowledge of substrate binding and catalysis. Features that distinguish DXP synthase from other ThDP-dependent enzymes can be exploited in the development of new anti-infective agents that target this first essential step in pathogen isoprenoid biosynthesis.

The focus of this work is the elucidation of substrate binding events to distinguish between ordered and ping-pong kinetic mechanisms. Here, we report the development of a catalytically active mutant DXP synthase possessing tryptophan fluorescence properties ideal for equilibrium substrate binding experiments. Additionally, we have performed detailed kinetic analyses using an unreactive pyruvate analog, methyl-acetylphosphonate (MAP), as a reversible inhibitor to elucidate substrate binding in the reaction catalyzed by DXP synthase. Our results indicate that D-GAP and pyruvate bind independently and reversibly to DXP synthase, suggesting the enzyme proceeds via a novel rapid equilibrium random sequential mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods**—Unless otherwise noted, all reagents were obtained from commercial sources. Spectrophotometric analyses were performed on a Beckman DU 800 UV-visible spectrophotometer. Primers were purchased from Integrated DNA Technologies. Tryptophan fluorescence studies were performed on an ISS Chronos spectrofluorimeter equipped with a 320-nm-long pass filter. *E. coli* wild-type DXP synthase and mutant Trp-76 DXP synthase were purified in the presence of ThDP as described previously (13). Molecular docking studies were performed using the program Autodock Vina (version 1.5.1) (16). Overproduction and Purification of IspC—*E. coli* BL21 cells harboring ispC-PET16b were grown to A₆₀₀ ~ 0.6 and induced with isopropyl 1-thio-β-D-galactopyranoside (100 μM) at 37 °C. Shaking was continued for 5 h, and the cells were harvested at 4 °C and stored overnight at ~20 °C. Thawed cells were suspended in protein purification buffer (25 mM Tris, pH 8.0, 400 mM NaCl, 10% glycerol), lysed by ultrasonication, and cell
debris was removed by centrifugation at 4 °C. The supernatant was incubated with nickel-nitriilotriacetic acid resin in 20 mM imidazole at 4 °C for 2 h, and IspC was eluted from the resin over a stepwise gradient of 5 to 500 mM imidazole. Fractions containing IspC (as determined by 10% SDS-PAGE) were combined and subjected to dialysis overnight at 4 °C against 1 liter of 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 10% glycerol. A second dialysis was carried out against 1 liter of 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM β-mercaptoethanol for an additional 4 h. After dialysis, protein concentration was determined using the Bio-Rad Protein Assay with bovine serum albumin as a standard (yield 33 mg/liter of culture). Protein was then flash frozen in liquid nitrogen and stored at −80 °C.

Site-directed Mutagenesis of DXP Synthase—Wild-type E. coli DXP synthase possesses four tryptophan residues (Trp-76, Trp-109, Trp-328, and Trp-618). Four triple mutants (Trp→Phe in each case) were constructed such that each mutant possessed a single tryptophan residue. Construction of the DXP synthase Trp-76 triple mutant (W109F, W328F, and W618F) used for equilibrium binding studies is described here. A similar approach was used to generate all other mutants. Mutagenesis mixtures contained 1× Pfu buffer (Stratagene), 200 μM dNTPs (Fermentas), 1 ng/μl template, 500 μM each primer, and 2 μl of Pfu-Turbo (Stratagene). The dxs-pET37b plasmid was used as the starting template, and the following primers (with the mutation underlined) were used to create the final dxs-pET37b construct containing W109F, W328F, and W618F mutations, leaving Trp-76 as the only reporter for tryptophan fluorescence spectroscopy. We sought to identify a tryptophan-bearing D-GAP and Pyruvate Bind Reversibly and Independently to DXP Synthase—Tryptophan fluorescence spectroscopy is widely used as a tool to determine substrate equilibrium binding constants and to examine protein conformation by observing changes in tryptophan fluorescence profiles. Wild-type DXP synthase possesses four tryptophan residues (Trp-76, Trp-109, Trp-328, and Trp-618) and an appreciable fluorescence signal. We sought to identify a tryptophan-bearing mutant enzyme suitable for reporting binding events in the active site. In addition, we required buffering conditions in which substrates are stable, DXP synthase activity is optimal, and collisional fluorescence quenching and nonspecific binding are minimized. A review of literature reports on DXP synthase reveals varying kinetic constants for the enzyme under a variety of conditions (6, 12, 13, 19, 20). Thus, a buffer optimization study was performed. The most efficient turnover of substrates was observed in HEPES buffer, pH 8.0 (K_m,GAP = 23.5 ± 1.7 μM, K_m,pyruvate = 48.7 ± 8.0 μM, k_cat = 153.6 ± 6.8...

RESULTS

D-GAP and Pyruvate Bind Reversibly and Independently to DXP Synthase—Tryptophan fluorescence spectroscopy is widely used as a tool to determine substrate equilibrium binding constants and to examine protein conformation by observing changes in tryptophan fluorescence profiles. Wild-type DXP synthase possesses four tryptophan residues (Trp-76, Trp-109, Trp-328, and Trp-618) and an appreciable fluorescence signal. We sought to identify a tryptophan-bearing mutant enzyme suitable for reporting binding events in the active site. In addition, we required buffering conditions in which substrates are stable, DXP synthase activity is optimal, and collisional fluorescence quenching and nonspecific binding are minimized. A review of literature reports on DXP synthase reveals varying kinetic constants for the enzyme under a variety of conditions (6, 12, 13, 19, 20). Thus, a buffer optimization study was performed. The most efficient turnover of substrates was observed in HEPES buffer, pH 8.0 (K_m,GAP = 23.5 ± 1.7 μM, K_m,pyruvate = 48.7 ± 8.0 μM, k_cat = 153.6 ± 6.8...
that binding of pyruvate to *R. capsulatus* DXP synthase induces a conformational change that promotes the binding of the second substrate \(\text{D-GAP}\) and that binding of \(\text{D-GAP}\) to the free enzyme is negligible (21) in the absence of pyruvate. This model predicts an exceedingly low \(K_d^{\text{pyruvate}}\) relative to \(K_m^{\text{pyruvate}}\). Similarly, the proposed ping-pong mechanism (15) predicts a low affinity of \(\text{D-GAP}\) for free enzyme (21). The Trp-76 DXP synthase mutant was used to test binding models. Interestingly, a binding constant of pyruvate was measured (Fig. 3A) that is comparable with its \(K_m^{\text{pyruvate}}\) \((K_d^{\text{pyruvate}} = 42.6 \pm 1.1 \mu M; K_m^{\text{pyruvate}} = 66.6 \pm 8.0 \mu M)\). A measurable binding constant suggests that pyruvate binding is reversible, a finding that is inconsistent with the previously proposed ordered mechanism (12). In addition, a binding constant for \(\text{D-GAP}\) that is comparable with its \(K_m^{\text{D-GAP}}\) was measured in the absence of pyruvate (Fig. 3B; \(K_d^{\text{D-GAP}} = 62.2 \pm 8.0 \mu M, K_m^{\text{D-GAP}} = 21.6 \pm 3.8 \mu M\)). This result indicates that \(\text{D-GAP}\) also undergoes reversible binding to the free enzyme. This finding is inconsistent with previously reported ordered or ping-pong mechanisms in which binding of \(\text{D-GAP}\) to the free enzyme is predicted to be negligible. Rather, this sort of substrate binding profile, where either substrate can bind reversibly and independently to free enzyme (E), is consistent with a rapid equilibrium, random sequential mechanism.

Substrate Affinities Are Minimally Affected by Co-substrate Binding—To investigate the influence of pyruvate on the binding of \(\text{D-GAP}\), as well as the influence of \(\text{D-GAP}\) on the binding of pyruvate, we performed tryptophan fluorescence experiments using MAP and the Trp-76 DXP synthase mutant. Both MAP and \(\beta\)-fluoropyruvate have been used previously as pyruvate mimics to study ThDP-dependent enzyme mechanisms (22–29). Unlike \(\beta\)-fluoropyruvate, which undergoes a catalytic
step to release carbon dioxide and subsequently eliminates fluoride following activation by ThDP (Fig. 4A), MAP is incapable of undergoing decarboxylation (Fig. 4B). Thus, MAP was selected as an appropriate pyruvate mimic for binding equilibrium studies carried out under non-turnover conditions.

The proposed ordered mechanism (Fig. 2A) predicts dramatic enhancement in D-GAP affinity for the E-MAP complex, whereas a ping-pong mechanism predicts low affinity of D-GAP for the PLThDP “pre-decarboxylation” complex that is possible upon binding of MAP to free enzyme (Fig. 4). In contrast, substrate affinities may or may not be influenced by binding of co-substrate in a random sequential mechanism.

Using Trp-76 DXP synthase, a binding constant of MAP was measured in the low μM range (Kd(MAP) = 12.0 ± 2.4 μM, Table 1, entry 4 and supplemental Fig. S5). To determine the influence of d-GAP on the binding of MAP, the Kd of MAP was measured in the presence of d-GAP. A comparable Kd was determined for MAP under these conditions (Table 1, entry 5; supplemental Table S2), suggesting the presence of GAP does not significantly influence MAP binding. The presence of subsaturating MAP only modestly influences d-GAP binding (Table 1, entry 2), whereas the Kd(GAP) at saturating concentrations of MAP (up to 1 mM) is not affected (supplemental Table S2). The lack of a significant enhancement in the affinity of d-GAP for DXP synthase in the presence of MAP argues against a strictly ordered kinetic mechanism in which pyruvate binds first and irreversibly (Fig. 2A). Taken together with the observed reversible binding of both natural substrates, these data are consistent with a rapid equilibrium, random sequential mechanism in which substrate affinities are unaffected by co-substrate binding (30).

D-GAP and Pyruvate Exhibit Substrate Inhibition—Additional mechanistic insight was obtained through detailed kinetic analysis of wild-type DXP synthase. DXP formation was monitored using the IspC-coupled reaction, and double-reciprocal plots of the natural substrates at varying concentrations were generated by plotting inverse initial velocities of DXP formation as a function of inverse substrate concentration at fixed concentrations of the second substrate in each case (Fig. 5). A rapid equilibrium, random sequential mechanism predicts a noncompetitive relationship for each case. Interestingly, the double-reciprocal plots shown in Fig. 5 exhibit apparently different trends at higher concentrations of the fixed second substrate. Under conditions where d-GAP is the varied substrate (Fig. 5B), the slopes exhibit a noncompetitive relationship at low concentration of pyruvate (12.3–36.8 μM) but appear to

![FIGURE 4. β-Fluoropyruvate and MAP shown as pyruvate mimics in a mechanism requiring ternary complex formation. A, following decarboxylation of β-fluoropyruvate, DXP synthase adopts a “post-decarboxylation” state. B, following formation of the phosphono-2-lactyl-ThDP (PLThDP) intermediate, the enzyme adopts a pre-decarboxylation state. E, ThDP-bound enzyme; Pyr, pyruvate; Pyr*, 2-hydroxyethyl-ThDP; G, D-GAP; E-Pyr-G, catalytically competent ternary complex.](image-url)
Random Sequential Mechanism in DXP Synthase Catalysis

Although incubation of DXP synthase with d-GAP in the absence of pyruvate does not result in new product formation or enzyme-dependent depletion of d-GAP (data not shown), a rapid equilibrium, random sequential mechanistic model considering the reversible substrate inhibition by d-GAP provides significantly improved curve fits in the non-linear regression analysis (Fig. 6B) and is supported by model discrimination analysis. The predicted $K_{i}^{d}$-GAP for the E-GAP complex is lower than the predicted $K_{i}^{pyruvate}$ for E-pyruvate complex, which is consistent with the observation that substrate inhibition by d-GAP appears more pronounced relative to substrate inhibition by pyruvate (Fig. 5 and supplemental Fig. S8).

Inhibition of DXP Synthase by Methylacetylphosphonate—β-Fluoropyruvate (24, 25) and phosphonate analogs of 2-oxo acids have been extensively used to study ThDP-dependent decarboxylating enzymes and their mechanisms (23, 28, 32–34). Pyruvate analogs β-fluoropyruvate and methylacetylphosphonate were evaluated as DXP synthase inhibitors to provide further support for a random sequential mechanism. For a random sequential mechanism, β-fluoropyruvate and MAP are predicted to behave as competitive inhibitors with respect to pyruvate but will exhibit a noncompetitive profile with respect to d-GAP. Initial rates of DXP formation were monitored using the IspC coupled assay at subsaturating, varied concentrations of one substrate, at fixed concentrations of the second substrate and in the presence of varying inhibitor concentrations (β-fluoropyruvate or MAP). The results (Fig. 7) show that MAP is a potent competitive inhibitor with respect to pyruvate (apparent $K_{i}^{MAP} = 715 \pm 108 \text{nM}$). When d-GAP is the varied substrate (Fig. 7), the trend is noncompetitive, as expected for a random sequential mechanism. A similar inhibition profile was observed for β-fluoropyruvate ($K_{i}^{β-fluoropyruvate} = 430 \pm 135 \text{nM}$; supplemental Fig. S9).

Inhibition Studies of DXP Synthase Reveal Important Binding Determinants—d-Glyceraldehyde was selected for inhibition studies as it is a commonly used d-GAP analog (6, 12, 19, 35). A random sequential kinetic mechanism predicts this analog to exhibit a competitive inhibition pattern with respect to d-GAP in a Lineweaver-Burk analysis and a noncompetitive profile with respect to pyruvate. However, d-glyceraldehyde exhibits a similar trend to MAP and β-fluoropyruvate, indicating competitive inhibition with respect to pyruvate (Fig. 8, apparent $K_{i}^{d-glyceraldehyde} = 3.2 \pm 0.4 \text{mM}$). It has been suggested that d-glyceraldehyde also acts as a substrate inhibitor competing for the pyruvate binding site at considerably higher concentrations (12). Clearly, removal of the phosphoryl moiety impacts the binding and recognition profile of this substrate analog, suggesting d-glyceraldehyde may not be an ideal alternative substrate for d-GAP in mechanistic studies of DXP synthase.

**DISCUSSION**

Despite its potential as an anti-infective target, selective inhibitors of DXP synthase are lacking, and reports on the kinetic mechanism are conflicting. The development of selective DXP synthase inhibitors requires detailed knowledge of the characteristics that distinguish this enzyme from other ThDP-dependent enzymes. The present study takes an important step toward understanding the mechanism of DXP synthase. We
have developed an active tryptophan-bearing DXP synthase mutant (Trp-76) suitable for measuring binding events at the enzyme active site and for discriminating ordered and ping-pong mechanisms on the basis of substrate binding and recognition. A detailed substrate binding analysis has revealed a surprisingly high affinity of D-GAP for free enzyme (\(K_d = 62.2 \pm 8.0\) \(\mu M\)) and reversible binding of pyruvate to free enzyme (\(K_d^{\text{pyruvate}} = 42.6 \pm 1.1\) \(\mu M\)). In both cases, binding constants are comparable with Michaelis-Menten constants for the Trp-76 DXP synthase mutant (\(K_m^{\text{D-GAP}} = 21.6 \pm 3.8\) \(\mu M\); \(K_m^{\text{pyruvate}} = 66.6 \pm 5.0\) \(\mu M\)). These observations are inconsistent with the proposed ordered mechanism which predicts negligible binding of D-GAP to free enzyme and a \(K_d^{\text{pyruvate}} \ll K_m^{\text{pyruvate}}\) (Fig. 2A). Similarly, a ping-pong mechanism (Fig. 2B) predicts negligible binding of D-GAP to free enzyme and irreversible activation of pyruvate with release of \(\text{CO}_2\). The observed reversible binding of pyruvate and D-GAP to free enzyme suggests an alternative, random substrate binding profile (Fig. 6). Molecular docking studies provide further support for this mechanistic model. Deinococcus radiodurans DXP synthase (Protein Data Bank code 2O1X) can easily accommodate both substrates near C2 of ThDP (Fig. 9). In addition, independent binding of either substrate to enzyme appears feasible and unobstructed by the presence of the other substrate.

MAP, a catalytically unreactive analog of pyruvate, was prepared to aid in elucidation of substrate binding order on the basis of D-GAP binding preferences. Our results demonstrate a reasonable affinity of D-GAP for the E-MAP complex as evidenced by a \(K_d^{\text{E-GAP}} = 19.8 \pm 4.4\) \(\mu M\), which is comparable with its Michaelis-Menten constant (\(K_m^{\text{E-GAP}} = 21.6\) \(\mu M\)). Interestingly, the affinity of D-GAP for DXP synthase is only subtly enhanced in the presence of subsaturating MAP, and this modest effect diminishes at higher concentrations of MAP. The affinity of MAP for DXP synthase is not enhanced in the presence of D-GAP. In principle, D-GAP could bind non-productively to the enzyme in the absence of pyruvate, giving rise to a measurable \(K_d\); however, the comparable affinity of D-GAP for the E-MAP complex is more compelling evidence in support of a rapid equilibrium, random sequential mechanism in which substrate affinities are not influenced by binding of co-substrate.

The distinction between the previously proposed mechanisms (12, 15) and the random sequential mechanism proposed here is difficult on the basis of kinetic analyses alone. The kinetic analysis presented here supports the notion that DXP synthase proceeds via a random sequential mechanism and highlights interesting differences with previously reported kinetic studies carried out on \(R.\ capsulatus\) DXP (12) and studies carried out on \(E.\ coli\) and \(H.\ influenzae\) DXP synthase (15). The results of this analysis suggest that pronounced substrate inhibition by D-GAP is observed as a result of a relatively high affinity of D-GAP for the E-GAP complex (supplemental Fig. S8).
observed inhibition by both natural substrates at reasonably low concentrations underscores the complexity of substrate equilibrium binding profiles and highlights a promiscuous active site of DXP synthase.

The discrepancies between the trends in the double-reciprocal analysis reported here and those reported previously (12, 15) may be explained by differences in reaction conditions. In our buffer optimization study, decreased turnover efficiency was observed when reactions were performed in Tris and citrate buffers reported previously for mechanistic studies of DXP synthase. In Tris buffer, $K_m$ for D-GAP values increased with increasing Tris concentration. This can be attributed to D,L-GAP instability in Tris, presumably a consequence of the reactivity of the aldehyde group of D,L-GAP toward the primary amine group of Tris (36). Citrate buffer was found to inhibit DXP synthase and IspC (supplemental Fig. S2), which is consistent with its properties as a magnesium chelator (37). Previous mechanistic reports describe kinetic experiments performed at higher varying substrate concentrations relative to concentration ranges of natural substrates in the present study. Presumably, these conditions were required to overcome the inhibitory effects and reactivity of these buffered systems. As substrate inhibition is most evident at lower concentrations of the varied substrate, it is anticipated that the subtle inhibitory effects of pyruvate and D-GAP are not observed under conditions where more pronounced inhibition by citrate buffer or removal of D-GAP by reaction with Tris buffer is taking place.

MAP exhibits a clear competitive mode of inhibition with respect to pyruvate, and a noncompetitive mode of inhibition with respect to D-GAP (Fig. 7), consistent with random sequential kinetic mechanism for DXP synthase. MAP is a well characterized inhibitor of many ThDP-dependent enzymes (28, 29). The observed potent inhibition of DXP synthase by this pyruvate analog raises the intriguing question about the utility of acetyl phosphonate derivatives as selective inhibitors targeting the unique conformations of this mechanistically distinct ThDP-dependent enzyme.

Interestingly, D-glyceraldehyde is competitive with pyruvate but not with D-GAP (Fig. 8). Although D-glyceraldehyde has been used previously as an alternative substrate for D-GAP (6, 12, 19), it has also been suggested that D-glyceraldehyde can compete for the pyruvate binding site as a substrate inhibitor at high millimolar concentrations (12). Here, we have shown that removal of the phosphoryl group of D-GAP changes the binding mode of the aldehyde such that it fails to compete for the D-GAP binding site. This result highlights the impact of the phosphoryl moiety on substrate recognition and binding and may underscore the importance of using the natural substrate D-GAP for mechanistic studies of this enzyme. In addition, this result further highlights the promiscuous active site of this enzyme, suggesting opportunities to develop selective inhibitors.

While this work was being prepared, a study describing single-molecule force spectroscopy for the identification of novel DXP synthase inhibitors was reported (14). The method allows
measurement of adhesion forces between immobilized substrates and enzyme. The authors suggest a possible dissociation of substrate recognition properties and substrate transformation properties that could permit the use of this catalytically less active nanosensor as a tool for identifying high affinity ligands without the need for substrate turnover. Interestingly, Sisquella et al. (14) reported binding of GAP tethered through the phos- phoryl moiety and enhancement of its affinity for the enzyme in the presence of soluble pyruvate. This is particularly interesting, in light of our finding that removal of the phosphoryl moiety inverts the binding mode of the acceptor aldehyde. It is possible that immobilization of DXP synthase effects changes in the active site that promote the binding of an unnatural immobilized substrate. Alternatively, it is conceivable that the immobilized monoanionic phosphate diester binds in a similar manner to d-GAP, suggesting that an acceptor substrate bearing a monoanionic substituent is sufficient to retain the natural binding mode.

The ThDP-dependent class of enzymes has been extensively studied. E. coli DXP synthase active site residues are conserved relative to other ThDP-dependent enzymes. These include His-49, proposed to play a role in proton transfer in a manner similar to that of His-30 in transketolase, and Glu-370, thought to be essential for cofactor activation (10, 38). In addition, R398A and R478A mutants were shown to be inactive (10, 38). Despite these similarities, DXP synthase exhibits a novel domain architecture and kinetic mechanism. The studies reported here demonstrate the independent, reversible binding of each natural substrate to DXP synthase. Coupled with kinetic analysis, these results suggest DXP synthase follows a rapid equilibrium, random sequential mechanism. This unexpected result raises intriguing questions about the role of d-GAP binding in steps leading to the generation of the nucleophilic C2α carbanion (Fig. 4), including the ionization and tautomerization states of ThDP and timing of pyruvate activation. In all other ThDP-dependent enzymes that catalyze decarboxylation via a ping-pong mechanism, the amino-pyrimidine ring of ThDP tautomerizes upon binding of the first substrate to promote the formation of a predecarboxylation complex (Fig. 4, LThDP) (28). In the unique case of DXP synthase, decarboxylation of pyruvate occurs only in the presence of the acceptor substrate d-GAP; it is not known whether d-GAP binding is required for tautomerization of the cofactor and formation of LThDP or if binding of d-GAP promotes decarboxylation of LThDP and formation of the C2α carbanion (Fig. 4).

Furthermore, in all of the other ThDP-dependent enzymes, the active sites display alternating catalytic activity in the common ping-pong mechanism used by this enzyme class (39); however, our finding that DXP synthase follows a random sequential mechanism raises interesting questions about the equivalency of active sites in this homodimer bearing a unique domain arrangement (10). Importantly, we have shown that DXP synthase exhibits striking differences in substrate binding from all other ThDP-dependent enzymes, which supports the idea that it will be possible to develop selective inhibitors of this distinct ThDP-dependent enzyme toward the development of anti-infective agents targeting early stage isoprenoid biosynthesis.

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FIGURE 9. Pyruvate (A) and d-GAP (B) docked to the active site of D. radio- durans DXP synthase. Each substrate binds to the active site without blocking access of the other (C).

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