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Revealing Donor Substrate-Dependent Mechanistic Control on DXPS, an Enzyme in Bacterial Central Metabolism

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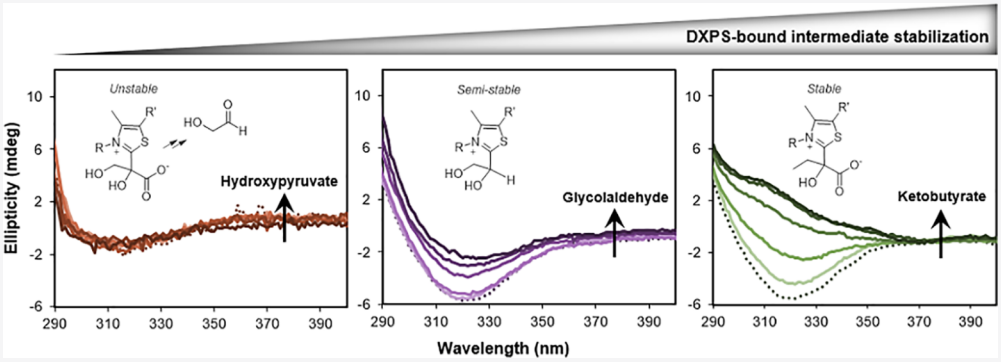

ABSTRACT: The thiamin diphosphate-dependent enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) catalyzes the formation of DXP from pyruvate (donor) and D-glyceraldehyde 3-phosphate (D-GAP, acceptor). DXPS is essential in bacteria but absent in human metabolism, highlighting it as a potential antibacterial drug target. The enzyme possesses unique structural and mechanistic features that enable development of selective inhibition strategies and raise interesting questions about DXPS function in bacterial pathogens. DXPS distinguishes itself within the ThDP enzyme class by its exceptionally large active site and random sequential mechanism in DXP formation. In addition, DXPS displays catalytic promiscuity and relaxed acceptor substrate specificity, yet previous studies have suggested a preference for pyruvate as the donor substrate when D-GAP is the acceptor substrate. However, such donor specificity studies are potentially hindered by a lack of knowledge about specific, alternative donor–acceptor pairs. In this study, we exploited the promiscuous oxygenase activity of DXPS to uncover alternative donor substrates for DXPS. Characterization of glycolaldehyde, hydroxypyruvate, and ketobutyrate as donor substrates revealed differences in stabilization of enzyme-bound intermediates and acceptor substrate usage, illustrating the influence of the donor substrate on reaction mechanism and acceptor specificity. In addition, we found that DXPS prevents abortive acetyl-ThDP formation from a DHEThDP carbanion/enamine intermediate, similar to transketolase, supporting the potential physiological relevance of this intermediate on DXPS. Taken together, these results offer clues toward alternative roles for DXPS in bacterial pathogen metabolism.

The bacterial enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) catalyzes the formation of DXP from pyruvate and D-glyceraldehyde 3-phosphate (D-GAP) in a thiamin diphosphate (ThDP)-dependent manner. The central metabolite DXP feeds into the biosynthesis of vitamins ThDP and pyridoxal phosphate as well as isoprenoid precursors isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) (Figure 1).[1,2] While DXP synthase (DXPS) is essential in many bacterial pathogens, it is absent in humans, highlighting its potential as an antibacterial drug target.[3−8] DXPS has unique structural and mechanistic features compared to other ThDP-dependent enzymes[9−14] that can be exploited to specifically target DXPS. Among these features are its large active site volume and unique domain arrangement compared to those of the related human ThDP-dependent enzymes transketolase (TK) and the E1 subunit of pyruvate dehydrogenase,[12,15−17] as well as a random sequential mechanism involving ternary complex formation.[9,14,18,19] Distinct from other ThDP-dependent enzymes, the first enzyme-bound intermediate, C2α-lactyl-ThDP (LThDP), is stabilized on DXPS in the absence of the acceptor substrates D-GAP and O_2.[10,13,20] D-GAP plays two roles on DXPS: as a trigger of LThDP decarboxylation upon binding to the E–LThDP complex and as an acceptor substrate in the subsequent carboligation step to produce DXP (Figure 1).[2,13,14] These structural and mechanistic features of DXPS have guided the development of inhibitors that display

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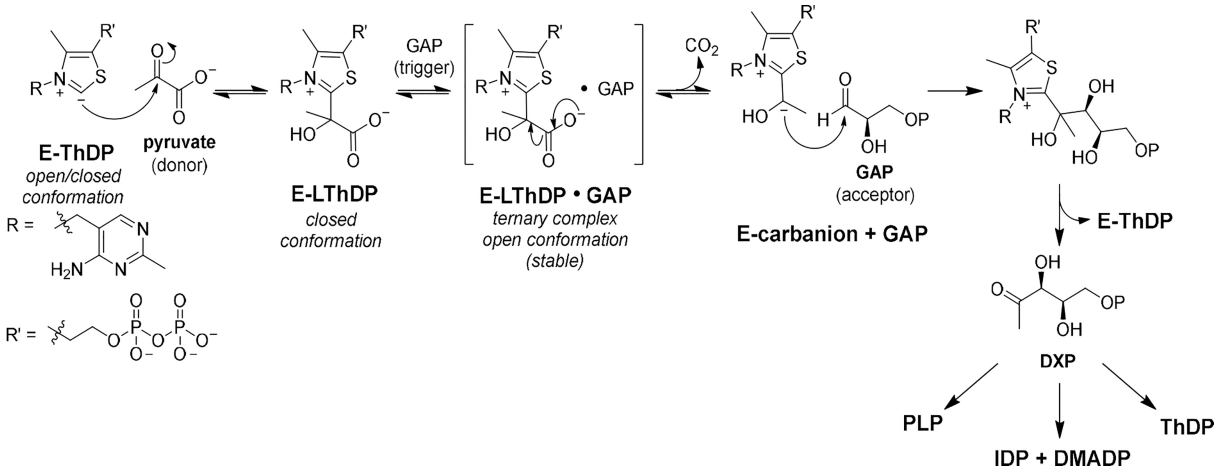

Figure 1. DXP formation on DXPS. Enzyme conformations at various steps in the reaction are indicated.[24,25] DXPS uses a gated mechanism in which LThDP is stabilized until a trigger induces decarboxylation.

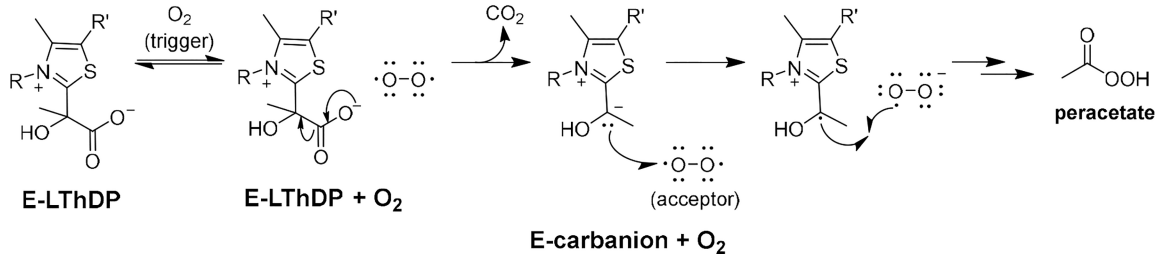

Figure 2. Oxygenase activity of DXPS. Oxygen acts as a trigger for LThDP decarboxylation and as an acceptor substrate on DXPS.

selective inhibition of DXPS over related ThDP enzymes.[7,8,21−23]

DXPS also possesses conformational flexibility and diversity,[10,19,24,25] as well as relaxed acceptor substrate specificity.[10−12,20,26−29] These characteristics, taken together with the role of DXPS in vitamin and isoprenoid biosynthesis, are reminiscent of multifunctional enzymes that are often found in central metabolism and existed in the last universal common ancestor.[30−34] This has led us to postulate that DXPS may also be multifunctional,[23] potentially having the capacity to catalyze diverse reactions in biology. Previous studies exploring the acceptor substrate specificity of DXPS have shown that the enzyme is capable of using structurally diverse acceptor substrates, including glyceraldehyde and other aldehydes with sterically demanding alkyl or aryl substituents, nitroso analogues, and O_2 (Figure 2).[10−12,20,26−29] In contrast, previous research to investigate donor substrate specificity has suggested pyruvate is the preferred donor in the presence of D-GAP as the acceptor.[27,28,34,35] In these studies, alternative donor substrate usage was assessed by detection of new products formed from α-ketoacids and D-GAP, and several potential donors were suggested to be inefficiently utilized under these conditions.[28,35] Given the minimal product formation observed in the presence of alternative α-ketoacids, we hypothesize that restricting the acceptor substrate to D-GAP in these studies limits our ability to detect a broader donor substrate specificity. It is possible that DXPS could use its unique gated mechanism to enable processing of distinct donor−acceptor pairs; D-GAP may not be the ideal acceptor substrate partner for other ketoacid donor substrates. Indeed, glycolaldehyde can be formed from hydroxypyruvate on DXPS,[36] whereas D-GAP appears to be inefficiently utilized in the presence of this donor.[28] Here, we have reasoned on the basis of the precedent for oxygenase activity on other carbanion-forming enzymes,[37−40] coupled with the observed DXPS-catalyzed oxidative decarboxylation of pyruvate,[20] that detecting oxygenase activity in the presence of alternative α-ketoacids may be a useful method for identifying donor substrates on DXPS.

In this study, we evaluated a series of ketoacids and aldehydes as potential donors for O_2 and show that DXP synthase utilizes hydroxypyruvate (HPA), ketobutyrate, and glycolaldehyde as alternative donor substrates in the oxygenase reaction. Interestingly, each donor displays a unique acceptor specificity and/or mechanistic profile. For example, pyruvate and HPA can use O_2 as an acceptor. However, unlike pyruvate, HPA does not undergo efficient carboligation with D-GAP, nor does DXPS stabilize its lactyl-like intermediate, β-hydroxylactyl-ThDP (β-OH-LThDP); HPA is readily converted to glycolaldehyde under anaerobic conditions. Additionally, DXPS avoids unproductive formation of acetyl-ThDP from dihydroxyethyl-ThDP (DHEThDP, the postdecarboxylation intermediate formed from HPA), which may support the physiological relevance of this intermediate. These results are significant because they indicate that DXPS couples substrate pair selection with mechanism, in which the donor substrate not only dictates acceptor substrate usage but also determines the mechanism of donor processing and its reactivity on DXPS. This apparent variable mechanistic control may offer additional support for the hypothesis that DXPS could be multifunctional.

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MATERIALS AND METHODS

General Methods. Unless otherwise noted, all materials were obtained from commercial sources. Chemicals, including substrates, were obtained from Sigma-Aldrich unless otherwise noted. Compound **3**, HEPES, and LB broth were purchased from Fisher; compound **9** was purchased from Fluka, and compound **4** from Alfa Aesar. *Escherichia coli* DXP synthase and *E. coli* IspC were overexpressed and purified as previously reported[9] with minor modifications. Lysis was performed using a Microfluidics (Westwood, MA) LM10 microfluidizer; the Ni-NTA batch bind was performed using 5 mM imidazole for 1 h, and the final dialysis was conducted using 1 L of a buffer lacking added β-mercaptoethanol. For DXPS overexpression, cells were grown at 37 °C for 1.5 h and then the temperature was decreased to 25 °C before induction and overnight growth. A Coy Laboratory Products (Grass Lake, MI) vinyl anaerobic chamber was used for all anaerobic experiments. A Tecan infinite M nano UV/visible plate reader situated inside the Coy chamber was used for anaerobic spectrophotometric analyses. Oxygen consumption was measured via an Oxytherm + respiration oxygen monitoring system from Hansatech Instruments Ltd. (Norfolk, U.K.). Anaerobic conditions for experiments conducted outside the chamber [e.g., circular dichroism (CD) and nuclear magnetic resonance (NMR)] were established in the Coy chamber, and then samples were transferred to anaerobic cuvettes (with septa lids) or NMR tubes (sealed with a layer of parafilm and a layer of vinyl tape). For CD studies, experiments were performed on an Aviv (Lakewood, NJ) 420 CD spectrometer.

Inhibition Assay. DXP formation was monitored using the previously described IspC-coupled assay with minor modifications.[9,41,42] Sample preparation and experiments were performed in the anaerobic chamber in a 96-well plate at ambient chamber temperature (25−29 °C). Reactions were initiated by simultaneous addition of pyruvate and D-GAP at $3K_M$ (90 and 93 μM, respectively). Each well contained 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8), 5 mM NaCl, 2 mM $MgCl_2$, 1 mM ThDP, 100 nM DXPS, 2 μM IspC, 400 μM NADPH, and 1 mM inhibitor. The disappearance of NADPH was monitored at 340 nm. Initial rates of NADPH depletion were used to estimate rates of DXP formation. The percent inhibition was calculated compared to a no inhibitor control. The standard deviation was calculated from three replicates.

Oxygenase Activity. To detect alternative donor usage by DXPS in the presence of O_2 as an acceptor, O_2 consumption was detected by an oxygen electrode. Unless otherwise noted, reactions included 5 μM DXPS and 1 mM substrate in 100 mM HEPES (pH 8), 100 mM NaCl, 2 mM $MgCl_2$, and 1 mM ThDP in a total volume of 350 μL. The electrode was calibrated daily at 25 °C, and solutions were prepared in the absence of substrate and equilibrated at 25 °C for a minimum of 2 min, until the O_2 signal stabilized. Reactions were initiated by the addition of 10 μL of a substrate by syringe (preincubated at 25 °C). The rate of O_2 consumption was determined by monitoring the concentration of O_2 over time at 25 °C. Reactions were performed in triplicate, and the standard deviation of the rate of O_2 depletion was calculated. Pseudo-Michaelis−Menten analysis was performed under the same conditions described above. Three separate Michaelis−Menten analyses were conducted; Michaelis−Menten curves were generated in each case and analyzed using Graphpad Prism. The standard deviation was calculated from the kinetic parameters of the three curves.

Steady State CD Analysis of DXPS-Bound Intermediates. Solutions were prepared and deoxygenated in a Coy anaerobic chamber for 30 min at ambient chamber temperature. Prior to removal from the chamber, solutions were transferred to a septa-capped cuvette (Starna Cells, 1-Q-10-ST-S). A DXPS + ThDP scan was performed with a 3 mL solution containing 40 μM DXPS in 50 mM HEPES (pH 8), 100 mM NaCl, 1 mM $MgCl_2$, and 0.2 mM ThDP. Scans were obtained at 4 °C scanning from 280 to 450 nm with a 1 nm step and a 3 s averaging time. The donor substrate was added via a gastight Hamilton syringe. The cuvette was inverted to mix and then scanned using the method described above. CD experiments were conducted at least twice for each donor substrate.

CD Detection of Formation of L-Erythrulose and Other Chiral Products. In each case, the solution was prepared and deoxygenated in a Coy anaerobic chamber for 30 min at ambient chamber temperature, after which it was transferred into a septa-capped cuvette (Starna Cells, 1-Q-10-ST-S). A DXPS + ThDP scan was performed with a solution containing 5 μM DXPS in 50 mM HEPES (pH 8), 100 mM NaCl, 1 mM $MgCl_2$, and 0.2 mM ThDP. The substrate was added once (25 mM HPA or 300 mM glycolaldehyde) via a Hamilton syringe. All scans used the following parameters: 250−400 nm with a 1 nm step and a 6 s averaging time. Scans were acquired at 25 °C for 1.5−2 h after the addition of the substrate, at which point the instrument and solution were cooled to 4 °C to slow the reaction, and an L-erythrulose standard (5 mM) was added. A final scan was acquired at 4 °C of the reaction mixture containing the L-erythrulose standard. To illustrate that L-erythrulose formation is slower at 4 °C (in the glycolaldehyde one-substrate reaction), one experiment was initiated at 4 °C for 60 min, at which point the temperature was increased to 25 °C and reaction progress monitored by CD spectroscopy for an additional 80 min. Experiments were performed in triplicate.

Detection of Aldehyde Formation. Aldehydes (acetaldehyde, propionaldehyde, and glycolaldehyde potentially formed from pyruvate, ketobutyrate, and HPA on DXPS) were detected using a coupled alcohol dehydrogenase (ADH) assay with ADH from *Saccharomyces cerevisiae* (Sigma).[36] For pseudo-Michaelis−Menten analysis of formation of glycolaldehyde from HPA, reaction mixtures contained 10 μM DXPS, 250 units/mL ADH, 50−5000 μM HPA, and 400 μM NADH in 200 μL of a solution containing 100 mM HEPES (pH 8), 100 mM NaCl, 2 mM $MgCl_2$, and 1 mM ThDP. Reaction mixtures were prepared in the anaerobic chamber and allowed to deoxygenate for 15 min before the reaction was initiated with the substrate and the absorbance recorded at 340 nm via the plate reader in the chamber (in 96-well plate format). All reactions were performed at chamber temperature in triplicate. Apparent K_m and k_{cat} values were calculated from fitting to the Michaelis−Menten curve in Graphpad Prism; error bars represent the standard deviation of three curves. Control reactions performed in the absence of DXPS were performed under aerobic conditions at 25 °C.

RESULTS

Evidence for Alternative Donor Substrate Binding and Utilization in the Presence of O_2. We hypothesized that acceptor substrate specificity on DXPS may vary according to the donor substrate and predicted other potential donors

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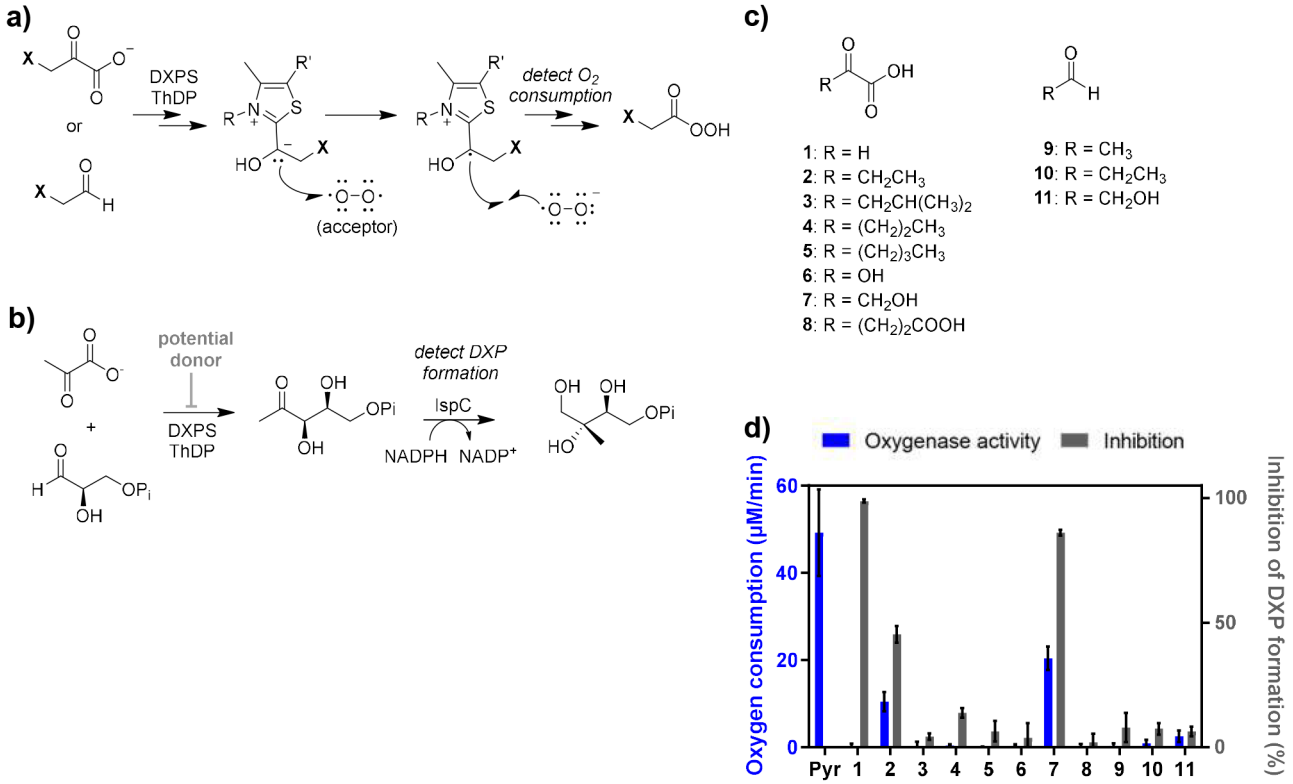

Figure 3. (a) Proposed use of oxygenase activity to detect carbanion formation from alternative donor substrates. (b) Use of inhibition of DXP formation to identify donor binding. (c) Compounds tested as potential alternative donor substrates for DXPS, including ketoacids (left) and aldehydes (right). (d) Evidence for alternative donor substrate binding and utilization. Comparison to oxygen consumption in the presence of pyruvate (Pyr) is also shown. The right axis (gray) shows inhibition of DXP formation. Conditions: anaerobic, ambient temperature, 100 nM DXPS, 2 μM IspC, 400 μM NADPH, 1 mM inhibitor. The left axis (blue) shows the rate of oxygen consumed. Conditions: 25 °C, 5 μM DXPS, 1 mM donor substrate. Error bars represent the standard deviation (n = 3).

could be detected more broadly via reaction with O_2 as a general electron acceptor. Thus, we measured O_2 consumption via an oxygen electrode in the presence of a variety α-ketoacids and aldehydes (Figure 3a,c). Under atmospheric conditions at 25 °C, robust DXPS-dependent O_2 consumption was observed in the presence of **2** (ketobutyrate) and **7** (hydroxypyruvate) (Figure 3d), both of which were previously deemed very poor substrates for DXPS in the presence of D-GAP.[28,35] DXPS-dependent O_2 consumption was also observed in the presence of **11** (glycolaldehyde) (Figure 3d), not previously studied as a donor for DXPS.

Because it is possible that some donors may react with ThDP on DXPS to form a lactyl-like intermediate that is stable in the presence of oxygen, and thus cannot be detected by O_2 consumption, we also evaluated potential donors as inhibitors of pyruvate-dependent DXP formation (Figure 3b). In this manner, we used inhibition of the DXP-forming reaction as evidence for donor binding in the active site. To ensure any effects are due to DXPS inhibition rather than an artifact of the coupled assay, we evaluated these compounds as inhibitors of IspC (Figure S1). For the majority of potential donor substrates tested, the results from the O_2 consumption and inhibition assays align (Figure 3d): substrates for the O_2-consuming reaction also inhibit DXP formation, whereas those that do not lead to O_2 consumption are also not inhibitors of DXP formation. However, **1** (glyoxylate) and **11** are two notable exceptions. Glyoxylate is the most potent inhibitor of the potential donors tested but is not a substrate for oxygenase activity under these conditions. Conversely, glycolaldehyde shows weak inhibitory activity but is a substrate for oxygenase activity on DXPS.

After detecting **1**, **2**, **7**, and **11** substrate reactivity on DXPS, we further characterized these four compounds. Given that glyoxylate is structurally similar to pyruvate but bears a reactive aldehyde group, we reasoned that it may act as either a donor or an acceptor substrate, or both, which could confound studies of its capacity to act as a donor. Indeed, ^{13}C NMR experiments conducted with glyoxylate using fully labeled [^{13}C]pyruvate as the donor substrate revealed multiple new products, indicating glyoxylate likely acts as an acceptor substrate (Figure S2). Due to the complex reactivity of glyoxylate on DXPS, its potential as an alternative donor was not further explored here. In this study, we performed more in-depth characterization of HPA, ketobutyrate, and glycolaldehyde to determine turnover efficiency in the presence of O_2 and obtain initial insights into the mechanism of donor processing and the preference for an acceptor substrate.

Glycolaldehyde. *Glycolaldehyde as an Oxygenase Substrate.* Oxygen consumption was used to examine glycolaldehyde as a donor substrate for O_2 (Figure 4a). The product of this reaction is tentatively assigned as 2-hydroxyethaneperoxic acid [**12** (Figure 4a)], analogous to peracetate formed in the presence of pyruvate and O_2.[20] O_2 consumption is observed in the presence of varying glycolaldehyde concentrations with an apparent k_{cat} of 1.8 ± 0.3 min^{-1} and an apparent K_m of 920 ± 120 μM under

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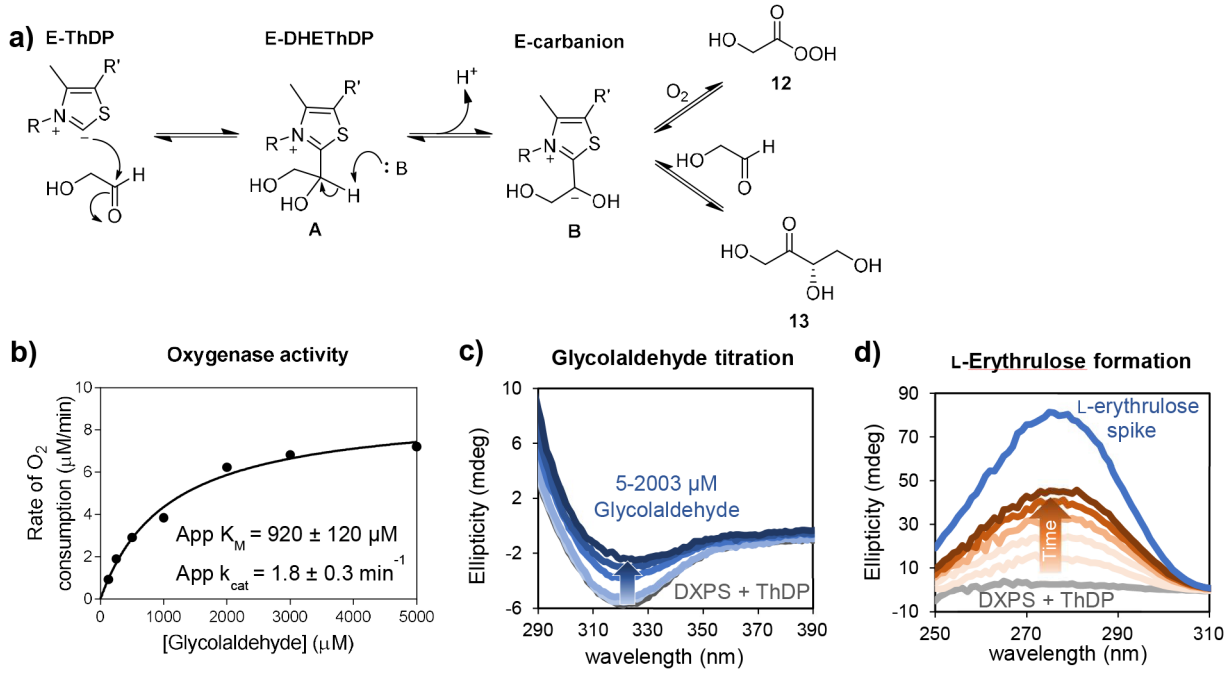

Figure 4. Characterization of glycolaldehyde as a substrate for DXPS. (a) Turnover of glycolaldehyde on DXPS. The product of oxygenase activity is tentatively assigned as the peroxyacid, **12**. (b) Oxygenase activity, representative analysis. Conditions: 25 °C, atmospheric O_2. The error represents the standard deviation (n = 3). (c) Representative CD titration of glycolaldehyde onto 40 μM DXPS, anaerobic, 4 °C (n = 3). (d) Representative CD trace of L-erythrulose (**13**) formation in the presence of 300 mM glycolaldehyde over time (1.75 h). After 1.5 h, the sample was cooled to 4 °C and 5 mM L-erythrulose standard was added (blue). Conditions: 5 μM DXPS, anaerobic, 25 °C (n = 3).

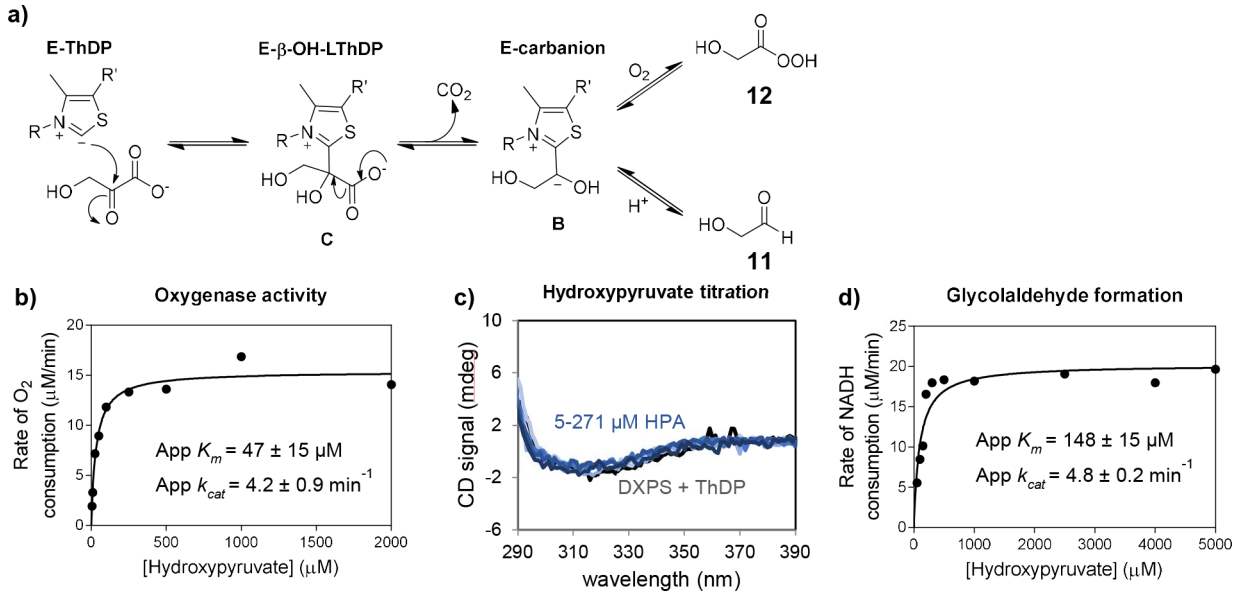

Figure 5. Characterization of HPA as a donor substrate for DXPS. (a) Turnover of HPA on DXPS to form glycolaldehyde, and the product of oxygenase activity that is tentatively assigned as the peroxyacid. (b) Oxygenase activity, representative Michaelis–Menten curve. Conditions: 25 °C, atmospheric O_2. Kinetic parameters determined are an average of three technical replicates. The error represents the standard deviation (n = 3). (c) Representative CD titration of HPA onto 40 μM DXPS, anaerobic, 4 °C. (d) Characterization of the aldehyde-forming reaction with HPA as the donor. A representative Michaelis–Menten curve is shown. Conditions: anaerobic, room temperature. The error indicates the standard deviation (n = 3).

atmospheric conditions (Figure 4a,b and Figure S3a). Oxidative decarboxylation of pyruvate under these conditions occurs at a comparable rate (apparent k_{cat} of 10 ± 8 min^{-1}) but with a higher affinity for pyruvate [apparent $K_m^{pyruvate}$ of 3 ± 2 μM (Figure S4)] than for glycolaldehyde.

CD Analysis of the Enzyme-Bound Intermediate. CD has previously allowed the visualization of LThDP formation and stabilization on DXPS in its random sequential mechanism to convert pyruvate to DXP.[13] Addition of glycolaldehyde to DXPS under anaerobic conditions (to eliminate oxygenase activity) causes depletion of the 4′-aminopyrimidine (AP)

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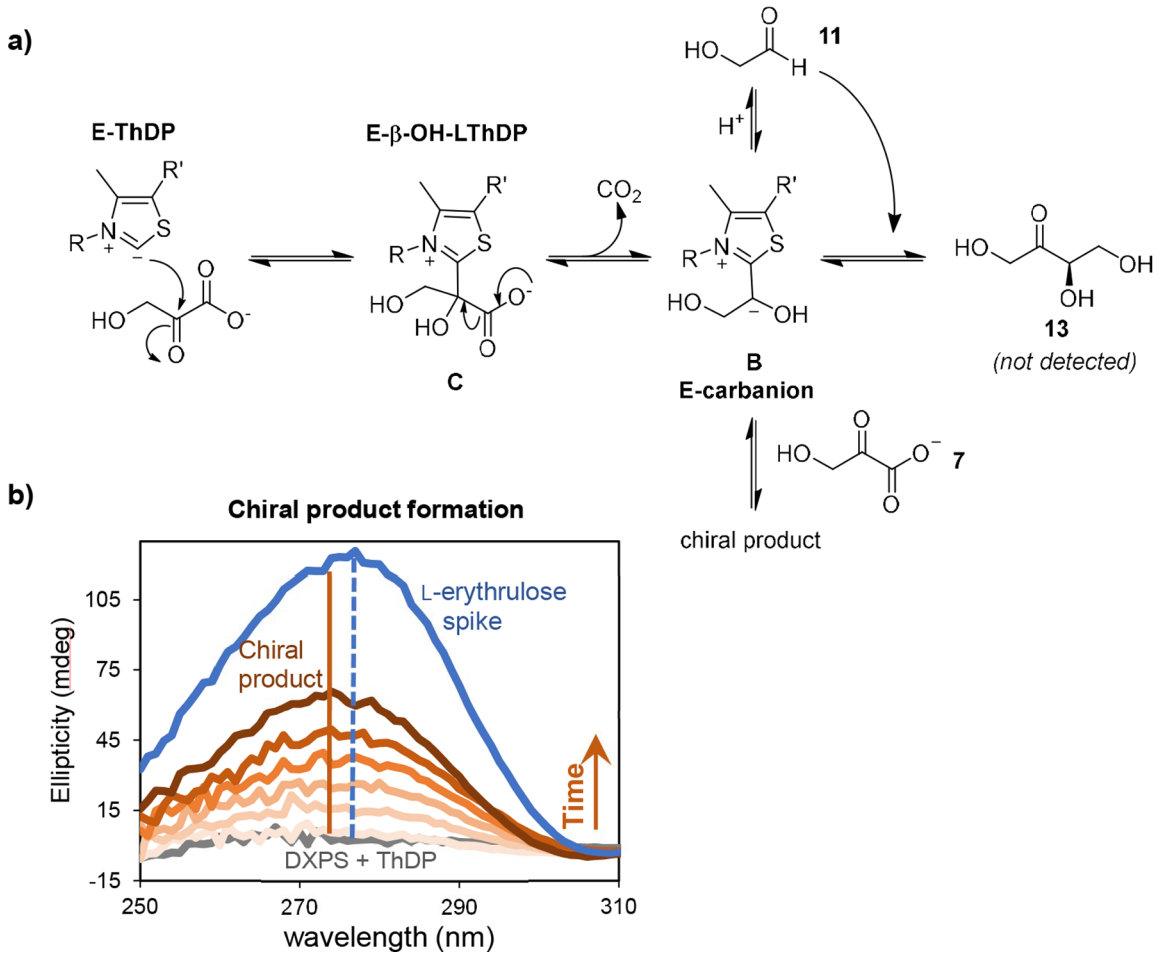

Figure 6. (a) Reaction of carbanion **B** to form a chiral product. (b) Representative CD trace (n = 2) of chiral product formation in the presence of 25 mM HPA over time (2 h). After 2 h, the mixture was cooled to 4 °C and 5 mM L-erythrulose standard (blue) was added. The CD_{max} for **13** (L-erythrulose, 278 nm, blue dotted line) is distinct from that of the new chiral product formed in the presence of HPA (274 nm, brown solid line). Conditions: 5 μM DXPS, anaerobic, 25 °C.

form (Figure 4c) of the E−ThDP complex, suggesting either intermediate formation or substrate turnover. While the AP signal does disappear with increasing concentrations of glycolaldehyde, there is a lack of inversion of the CD signal (Figure 4c), in contrast to the observed accumulation of a stable LThDP signal from pyruvate.[20] This could be due to the low molar ellipticity of **A**, the reversibility of intermediate **A** formation (semistable intermediate), or turnover of **A** to form a product. L-Erythrulose (**13**) is an expected product from a "one-substrate" reaction, where two molecules of glycolaldehyde are utilized as the donor and acceptor.[43,44] Thus, we used CD and NMR to investigate turnover of **A** to form erythrulose.

CD- and NMR-Based Detection of Formation of L-Erythrulose from Glycolaldehyde. CD was used to determine if DXPS can catalyze the formation of erythrulose from two molecules of glycolaldehyde, as previously described for TK.[43] Addition of 300 mM glycolaldehyde to DXPS at 25 °C results in a positive CD signal with a λ_{max} of 278 nm, which is consistent with previous characterizations of L-erythrulose.[43,45] A standard of L-erythrulose added to the sample increases the magnitude of the product signal (Figure 4d). L-Erythrulose formation on DXPS was further confirmed by NMR analysis, with the new product showing characteristic peaks at 4.57 and 4.46 ppm (Figure S3b).[44] L-Erythrulose formation is minimized at 4 °C (Figure S3c), conditions under which the stability of intermediate **A** was measured. Thus, it is unlikely that erythrulose formation contributes to the CD profile observed upon addition of low concentrations of glycolaldehyde to DXPS (Figure 4c).

Hydroxypyruvate (HPA). *HPA as an Oxygenase Substrate.* Oxygen consumption was measured over a range of HPA concentrations (Figure 5b) and DXPS concentrations (Figure S5a) to demonstrate the HPA and DXPS dependence of O_2 depletion, presumably forming **12** (Figure 5a). The maximum rate of O_2 consumption under atmospheric conditions at 25 °C is achieved at HPA concentrations between 250 and 2000 μM O_2 (Figure 5b). The apparent k_{cat} of this reaction is 4.2 ± 0.9 min^{-1}, and the apparent K_m^{HPA} is 47 ± 15 μM (Figure 5b), which is comparable to the $K_m^{pyruvate}$ in the reaction with D-GAP to form DXP ($K_m^{pyruvate}$ = 49 ± 5 μM).[20]

CD Analysis of the Enzyme-Bound Intermediate. CD was also used to detect the formation and subsequent depletion of the postdecarboxylation intermediate [**B** (Figure 5a)], the DHEThDP carbanion/enamine, formed from HPA on TK.[46,47] Thus, we conducted a CD analysis of the reaction of HPA on DXPS under anaerobic conditions lacking a known trigger of decarboxylation, to determine whether DXPS stabilizes a pre- or postdecarboxylation intermediate formed from HPA [**C** or **B**, respectively (Figure 5a)]. Interestingly,

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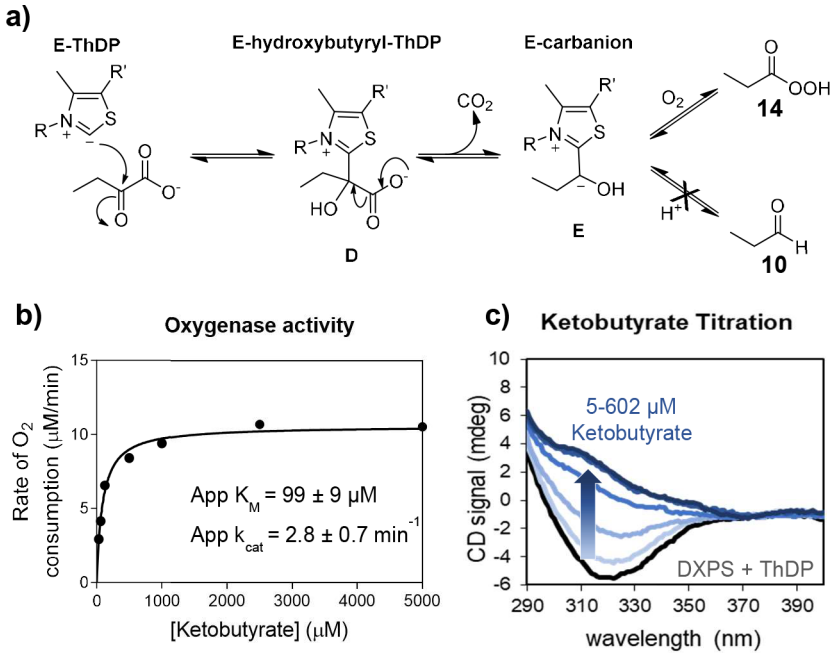

Figure 7. Characterization of ketobutyrate as a donor substrate for DXPS. (a) Turnover of ketobutyrate on DXPS. The product of oxygenase activity is tentatively assigned as the peroxyacid, **14**. (b) Oxygenase activity, representative Michaelis−Menten curve. Conditions: 5 μM DXPS, 25 °C, atmospheric O_2. The error represents the standard deviation (n = 3). (c) Representative CD titration of ketobutyrate onto 40 μM DXPS, anaerobic, 4 °C.

upon addition of HPA (5−271 μM) to DXPS (40 μM) under anaerobic conditions, there is no buildup of a CD signal to indicate accumulation of intermediates **C** or the protonated form of **B** (Figure 5c), DHEThDP [**A** (Figure 4a)], suggesting that either HPA does not undergo activation to form a predecarboxylation intermediate (**C**) on DXPS in the absence of O_2 or **C** forms but is unstable on DXPS under this condition relative to the DXPS-bound intermediates formed in the presence of the other donors in this study.

Analysis of Glycolaldehyde Formation. Given that a stable predecarboxylation intermediate (**C**) does not accumulate on DXPS in the presence of HPA under anaerobic conditions, we hypothesized that HPA may react with the E−ThDP complex to form intermediate **C**, but the barrier to decarboxylation is sufficiently low such that β-OH-LThDP (**C**) buildup is not observed, and decarboxylation to form carbanion intermediate **B** readily occurs. In this case, formation of glycolaldehyde would provide evidence of carbanion formation, indicating processing of HPA via protonation of the carbanion intermediate. Thus, DXPS-dependent formation of glycolaldehyde from HPA was measured under anaerobic conditions using a coupled enzyme assay with DXPS and alcohol dehydrogenase (ADH), which consumes glycolaldehyde in an NADH-dependent manner (Figure S6a). Indeed, HPA is turned over to form glycolaldehyde at 25 °C, with a K_m^{HPA} of 148 ± 15 μM and a k_{cat} of 4.8 ± 0.2 min^{-1} (Figure 5d), and at 10 °C (data not shown).

Taken together, these studies of HPA reactivity on DXPS suggest a distinct mechanism and acceptor substrate specificity for turnover of HPA compared to those of pyruvate. In contrast to pyruvate, HPA appears to undergo activation and decarboxylation without a requirement for ternary complex formation, and the corresponding carbanion (**B**) does not undergo carboligation with D-GAP but is instead readily protonated following decarboxylation. Pyruvate can react with a variety of acceptor substrates,[11,12,20] but in contrast to HPA, pyruvate is not readily converted to acetaldehyde under conditions where HPA is converted to glycolaldehyde.

CD- and NMR-Based Detection of Formation of a Chiral Product from HPA. We reasoned it is possible for DXPS to catalyze formation of L-erythrulose in the presence of HPA (Figure 6a), similar to the one-substrate reaction that occurs on DXPS in the presence of glycolaldehyde (Figure 4a,d). We rationalized this on the basis of the fact that decarboxylation of **C** leads to the same reactive carbanion (**B**), together with the observation that glycolaldehyde is a likely product of HPA decarboxylation on DXPS and would be available to react with the carbanion [**B** (Figure 6a)]. There is also precedence for this type reaction by TK in the presence of HPA.[44,48] CD analysis indicates the DXPS-dependent formation of a new chiral product in the presence of HPA with a CD_{max} at 274 nm (Figure 6b), distinct from the CD signal produced by L-erythrulose (278 nm). NMR analysis also supports formation of a new product that can be distinguished from L-erythrulose (Figure S5b), differentiating this reaction from the TK-catalyzed reaction.[48]

HPA Does Not Inhibit DXPS in a Time-Dependent Manner. On some, but not all, ThDP-dependent enzymes (e.g., pyruvate decarboxylase, acetohydroxyacid synthase, and pyruvate:ferredoxin oxidoreductase, but not TK), HPA acts as a mechanism-based inhibitor, undergoing elimination to release water to form acetyl-ThDP (Figure S7a).[49−51] HPA inhibits DXP formation (Figure 3); thus, we evaluated the time dependence of inhibition. DXPS activity is not suppressed in a time-dependent manner (over the course of 0.5−20 min) in the presence of 0.5 mM HPA (Figure S7b). In addition, acetate, the product of acetyl-ThDP hydrolysis,[52−56] was not detected by ^{1}H NMR [5 μM DXPS, 25 mM HPA, 25 °C (data not shown)]. Taken together, these results suggest that HPA does not cause time-dependent inactivation of DXPS via

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acetyl-ThDP formation. This is reminiscent of TK, where HPA is a substrate inhibitor[57−59] and does not display time-dependent inhibition.[47,50,60]

Ketobutyrate. *Ketobutyrate as an Oxygenase Substrate.* Ketobutyrate-dependent O_2 depletion in the presence of DXPS is observed, indicating ketobutyrate is a donor substrate for DXPS when O_2 is available to act as an acceptor substrate (Figure S8a). This oxygenase activity (Figure 7a) was studied by measuring the rate of O_2 consumption at varied ketobutyrate concentrations under atmospheric conditions. The apparent k_{cat} is 2.8 ± 0.7 min^{-1}, and the apparent $K_m^{ketobutyrate}$ is 99 ± 9 μM (Figure 7b), indicating reasonable affinity of ketobutyrate for DXPS.

CD Analysis of the Enzyme-Bound Intermediate. Upon addition of ketobutyrate to DXPS under anaerobic conditions, a positive CD signal at approximately 313 nm emerges (Figure 7c), indicating a shift of the ThDP cofactor to the 1′,4′-iminopyrimidine (IP) form, which is consistent with stabilization of a predecarboxylation intermediate [**D** (Figure 7a)], as is observed with pyruvate as the donor.[13] Notably, aeration of the ketobutyrate-saturated sample leads to depletion of the positive CD signal at 313 nm (Figure S8b), suggesting reaction of **D** with O_2. A similar observation was made upon aeration of samples containing LThDP.[20] The apparent K_D of ketobutyrate is 16 ± 9 μM under anaerobic conditions (Figure S8c), which is comparable to the apparent $K_D^{pyruvate}$ (45 ± 2 μM).[20]

Analysis of Propionaldehyde Formation. LThDP, formed from pyruvate, is stabilized on DXPS and is not readily converted to acetaldehyde via protonation of the carbanion.[61] Given that DXPS appears to stabilize the lactyl-like intermediate [**D** (Figure 7a)] formed from ketobutyrate, in a manner similar to stabilization of LThDP formed from pyruvate, it was expected that **D** may also not be readily converted to the corresponding aldehyde formed via protonation of the carbanion (**E**). Detection of propionaldehyde formation using the ADH-coupled assay (Figure S6b) revealed that aldehyde formation in the presence of ketobutyrate is inefficient, similar to acetaldehyde formation in the presence of pyruvate (Figure S6c,d).

DISCUSSION

DXPS has broad acceptor substrate specificity and catalytic promiscuity;[11,12,20] however, previous studies conducted using D-GAP as the acceptor substrate have suggested that DXPS prefers pyruvate as a donor substrate.[28,35] In the study presented here, we considered the possibility that using D-GAP as the sole acceptor in donor substrate specificity studies precludes the discovery of alternative donor substrates for DXPS. We utilized the recently characterized oxygenase activity of DXPS as a tool to detect alternative donor substrate usage and have shown that from a small collection of α-ketoacids and aldehydes, HPA, ketobutyrate, and glycolaldehyde readily act as donor substrates in the presence of O_2 as the acceptor substrate. Ketobutyrate and HPA display reasonable affinities for the enzyme when O_2 is the acceptor, comparable to pyruvate. Although kinetic parameters for these two donors in the presence of D-GAP were not determined previously, they were reported to display exceptionally low rates of product formation (<10% compared to DXP formation) and thus deemed poor substrates for DXPS.[28,35]

Comparing ketobutyrate, HPA, and glycolaldehyde as donor substrates, we found interesting distinctions among these substrates began to emerge. According to our CD results, ketobutyrate and pyruvate display similar affinity for DXPS and similar reactivity. Both predecarboxylation intermediates are stabilized on the enzyme under anaerobic conditions; neither pyruvate nor ketobutyrate reacts to form the corresponding aldehydes, and both are turned over in the presence of O_2. Similarly, glycolaldehyde is turned over in the presence of O_2. In addition, a second molecule of glycolaldehyde acts as the acceptor substrate in a one-substrate reaction in which L-erythrulose is formed on DXPS (25 °C, 5 μM DXPS).[43,44] CD analysis to detect the enzyme-bound intermediate in the presence of glycolaldehyde (4 °C, 40 μM DXPS) reveals a semistable signal with small changes upon addition of glycolaldehyde, which could be due to the reversibility of intermediate formation or glycolaldehyde turnover to form L-erythrulose. Previous work on TK supports the hypothesis that the lack of inversion of the CD signal is due to the reversibility of the reaction. When D-xylulose 5-phosphate (a reversible donor) was visualized on TK by CD, disappearance of the AP form of the cofactor was observed to produce a semistable signal without inversion of the signal to indicate DHEThDP buildup; however, when HPA (an irreversible donor due to CO_2 release) was visualized on TK, an inverted, stable CD signal was observed, indicative of DHEThDP buildup.[62] An equilibrium between DHEThDP (**A**) and the carbanion (**B**) that prevents inversion of the CD signal (as only **A** would be visualized by CD) cannot be ruled out; among ThDP-dependent enzymes, it is thought that some can stabilize the carbanion/enamine intermediate.[63] Finally, while the small changes in CD signal upon addition of glycolaldehyde may be explained by turnover of glycolaldehyde to form L-erythrulose, formation of this product is challenging to detect under the conditions used to observe DXPS-bound intermediates. Given this one-substrate reaction is minimized at 4 °C, and taking into account the increased background in the L-erythrulose signal region (278 nm) from the high DXPS concentration (40 μM) required to observe enzyme-bound intermediates, it is not surprising that L-erythrulose formation by CD is not observed under these conditions. The conversion of glycolaldehyde to L-erythrulose on DXPS is also unlikely to be physiologically relevant, given the high concentrations of glycolaldehyde required.

HPA appears to be unique among DXPS donor substrates in that, even under anaerobic conditions, its predecarboxylation intermediate (**A**) is unstable and does not accumulate on DXPS. Previous work by the Copley group has shown that DXPS can use HPA and H^+ to form glycolaldehyde under aerobic conditions, where O_2 is also present (K_m^{HPA} of 50 μM, compared to $K_m^{pyruvate}$ in DXP formation of 96 μM).[36] Here, we determined that this glycolaldehyde-forming activity occurs even in the absence of O_2, suggesting that this reaction does not require ternary complex formation and/or use of a known trigger for decarboxylation to proceed on DXPS. Intriguingly, this suggests that DXPS controls the chemistry of HPA turnover by a mechanism different from pyruvate turnover, perhaps similar to that of TK which is also known to process HPA to DHEThDP in the absence of an acceptor substrate.[47,64]

CD and NMR analyses indicate the chiral product formed under conditions of high HPA concentrations is distinct from L-erythrulose.[11,13] It is plausible that the tautomeric forms of HPA and its instability[65] contribute to its reactivity on DXPS at high concentration and are unlikely to be physiologically

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relevant. In contrast to DXPS, TK catalyzes formation of L-erythrulose from HPA, a reaction in which HPA is proposed to undergo conversion to glycolaldehyde, which remains in the active site and acts as the acceptor substrate for a second HPA donor substrate.[48] Aside from the distinct HPA-dependent reaction we observed here, DXPS appears to process HPA in a manner that is similar to that of transketolase, including conversion of HPA to glycolaldehyde and prevention of the abortive side reaction to form acetyl-ThDP. Time-dependent inactivation by HPA via acetyl-ThDP formation is observed on other ThDP-dependent enzymes, including pyruvate decarboxylase (PDC), acetohydroxyacid synthase (AHAS), and pyruvate:ferredoxin oxidoreductase (PFOR).[49−51] Pyruvate dehydrogenase (PDH) is not inhibited by HPA, consistent with the observation that HPA is also not a substrate for PDH and likely has a low affinity for the PDH active site.[49,50,66] Like DXPS, TK does not appear to catalyze elimination of water from β-OH-LThDP [intermediate **C** (Figure 5)] to form acetyl-ThDP. It has been hypothesized that this abortive acetyl-ThDP formation does not occur on TK as this would waste the physiologically important intermediate DHEThDP carbanion/enamine [intermediate **B** (Figure 5)] formed from both D-xylulose 5-phosphate and D-sedoheptulose 7-phosphate.[43,50,67] Conversely, other ThDP enzymes that do catalyze this dehydration reaction in the presence of HPA have not been shown to catalyze reactions involving the DHEThDP carbanion/enamine intermediate in physiologically relevant reactions *in vivo*. On the basis of these previously reported trends, it is conceivable that DXPS prevents unproductive formation of acetyl-ThDP from HPA because the DHEThDP carbanion/enamine intermediate may be a naturally occurring intermediate on DXPS under some physiological conditions. Further studies are required to test this hypothesis.

This work offers a new tool for studying donor substrate usage on DXPS by exploiting its promiscuous oxygenase activity. Overall, our studies of three different donors detected by this method offer an important biochemical basis for the capacity of DXPS to display alternative reactivities and mechanisms and enhance our understanding of DXPS promiscuity; it is now clear that DXPS can use multiple donor, trigger, and acceptor substrates. The physiological relevance of alternative activities of DXPS is not yet understood. However, the ability to catalyze multiple reactions, using distinct donor−acceptor pairs and processing by distinct mechanisms, could support a hypothesis of DXPS multifunctionality.[30−33] Studying the mechanism by which DXPS controls its alternative chemistries is of interest, as is further investigation of the potential physiological relevance of alternative activities. The conformational flexibility, substrate and catalytic promiscuity, and the capacity of DXPS to tune its chemistry are potentially significant, as these characteristics of DXPS could imply alternative functions of this enzyme in bacterial metabolism.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.1c00019.

General methods, controls to assess inhibition of IspC NMR analysis of glyoxylate as an acceptor substrate for DXPS NMR analysis of L-erythrulose formation, aeration of enzyme-bound intermediates (CD), apparent K_D determination, summary of donor substrate reactivity on DXPS (Table S1), inhibition of IspC by potential alternative donors (Figure S1), NMR analysis of glyoxylate as an acceptor substrate for DXPS (Figure S2), glycolaldehyde reactivity on DXPS (Figure S3), characterization of DXPS oxygenase activity in the presence of pyruvate (Figure S4), HPA reactivity on DXPS (Figure S5), ADH substrate specificity and detection of aldehyde formation on DXPS (Figure S6), assessing time-dependent inactivation by HPA (Figure S7), and characterization of ketobutyrate as a donor substrate on DXPS (Figure S8) (PDF)

Accession Codes

E. coli DXP synthase, Uniprot P77488; NCBI, Q0TKM1; *E. coli* MEP synthase, Uniprot P45568; NCBI, ATZ31749.

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C.L.F.M. and M.L.J. conceived and designed the study, interpreted results, and prepared the manuscript. M.L.J. designed and conducted all experiments and data analysis.

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Notes

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■ ABBREVIATIONS

DXPS, 1-deoxy-D-xylulose 5-phosphate synthase; D-GAP, D-glyceraldehyde 3-phosphate; ThDP, thiamin diphosphate; IDP, isopentenyl diphosphate; DMADP, dimethylallyl diphosphate; TK, transketolase; LThDP, C2α-lactylthiamin diphosphate; HPA, hydroxypyruvate; DHEThDP, α,β-dihydroxyethylThDP; AP, 4′-aminopyrimidine tautomer of ThDP; IP, 1′,4′-iminopyrimidine tautomer of ThDP; β-OH-LThDP, β-hydroxyactylThDP; ADH, alcohol dehydrogenase.

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