JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

Observation of Thiamin-Bound Intermediates and Microscopic Rate Constants for Their Interconversion on 1-Deoxy-D-xylulose 5-Phosphate Synthase: 600-Fold Rate Acceleration of Pyruvate Decarboxylation by D-Glyceraldehyde-3-phosphate.

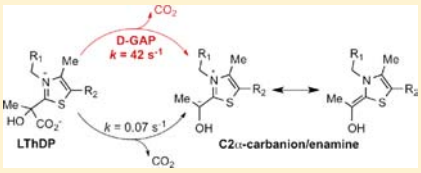
Hetalben Patel,[†] Natalia S. Nemeria,[†] Leighanne A. Brammer,[‡] Caren L. Freel Meyers,*[,‡] and Frank Jordan*[,†]

[†]Department of Chemistry, Rutgers University, Newark, New Jersey 07102, United States

[‡]Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, United States

Supporting Information

ABSTRACT: The thiamin diphosphate (ThDP)-dependent enzyme 1-deoxy-D-xylulose 5-phosphate (DXP) synthase carries out the condensation of pyruvate as a 2-hydroxyethyl donor with D-glyceraldehyde-3-phosphate (D-GAP) as acceptor forming DXP. Toward understanding catalysis of this potential anti-infective drug target, we examined the pathway of the enzyme using steady state and presteady state kinetic methods. It was found that DXP synthase stabilizes the ThDP-bound predecarboxylation intermediate formed between ThDP and pyruvate (C2α-lactylThDP or LThDP) in the absence of D-GAP, while addition of D-GAP enhanced the rate of decarboxylation by at least 600-fold. We postulate that decarboxylation requires formation of a ternary complex with both LThDP and D-GAP bound, and the central enzyme-bound enamine reacts with D-GAP to form DXP. This appears to be the first study of a ThDP enzyme where the individual rate constants could be evaluated by time-resolved circular dichroism spectroscopy, and the results could have relevance to other ThDP enzymes in which decarboxylation is coupled to a ligation reaction. The acceleration of the rate of decarboxylation of enzyme-bound LThDP in the presence of D-GAP suggests a new approach to inhibitor design.



INTRODUCTION

The enzyme 1-deoxy-D-xylulose 5-phosphate (DXP) synthase generates the first crucial intermediate in the biosynthesis of both thiamin and pyridoxal as well as in isoprenoid biosynthesis essential in human pathogens.[1−3] DXP synthase uses thiamin diphosphate (ThDP) as coenzyme and pyruvate and D-GAP as substrates. The reaction catalyzed by DXP synthase combines aspects of decarboxylase and carboligase enzymes. The chemical mechanism of the first step is likely to parallel typical ThDP-dependent pyruvate and other 2-oxoacid decarboxylase enzymes, the pyruvate forming a predecarboxylation covalent intermediate with ThDP (C2α-lactylThDP or LThDP) followed by decarboxylation to an enamine/C2α-carbanion/C2α-hydroxyethylidene-ThDP, which undergoes carboligation with the aldehyde functional group of D-GAP to form DXP (Scheme 1 right).

Previous mechanistic studies of DXP synthase were performed under steady-state conditions.[4−7] We report the first study of DXP synthase using tools capable of observing and determining the time-resolved behavior of ThDP-bound covalent intermediates (Scheme 1, right): stopped-flow circular dichroism (CD) and chemical quench followed by NMR detection. The CD method can monitor events on the enzyme itself, the NMR method monitors ThDP-bound intermediates after release from the enzyme (fortuitously, all the major ones are stable in acid), and is also useful to provide positive identification of the species seen in the CD spectrum. The CD studies take advantage of (a) identification of an electronic absorption for the 1′,4′-iminopyrimidine (IP form, Scheme 1 left) of ThDP at 300−310 nm[8] and (b) demonstration on 10 ThDP enzymes so far that at pH values at or above the pK_a of the 4′-aminopyrimidinium form (APH$^+$) of ThDP, the IP tautomer predominates in ThDP-bound covalent intermediates where the C2α-carbon carries four substituents.[9]

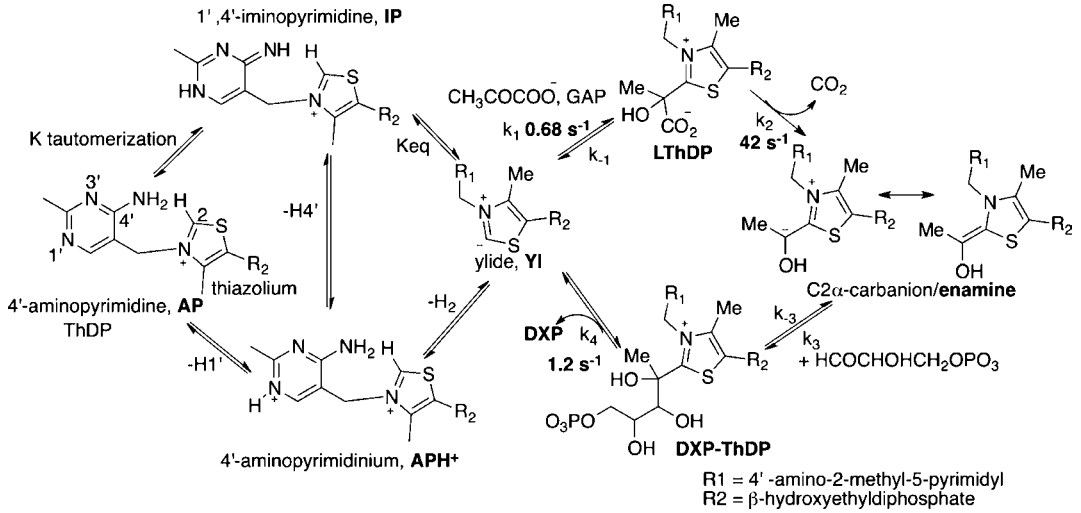
EXPERIMENTAL PROCEDURES

Materials. ThDP, pyruvate, yeast alcohol dehydrogenase, nicotinamide adenine dinucleotide, 2,6-dichlorophenolindophenol (DCPIP), D,L-GAP, and 1-deoxy-D-xylulose 5-phosphate (DXP) were from Sigma-Aldrich (St. Louis, MO). HEPES and dithiothreitol were from USB (Cleveland, OH). DXP synthase was purified as reported previously.[7]

Received: July 25, 2012
Published: October 16, 2012

   dx.doi.org/10.1021/ja307315u | J. Am. Chem. Soc. 2012, 134, 18374−18379

Scheme 1. Presteady State Detection of Forward Rate Constant for Individual Steps in DXP Synthase Catalytic Cycle at 6 °C with Both Pyruvate and GAP Present in LThDP Formation[a]



[a] k_1 is formation of LThDP; k_2 is decarboxylation to enamine; k_3 is carboligation with D-GAP to provide DXP~ThDP; k_4 is release of DXP product. CD studies assume that LThDP and DXP~ThDP when bound to DXP synthase are both in the IP tautomeric form while NMR can differentiate them by the method of Tittmann et al.[14] The decarboxylation rate constant 4.2×10^1 s^{-1} pertains to k_2.

Activity Measurement of DXP Formation Using Coupled Enzyme (IspC). DXP synthase activity was measured spectrophotometrically using pyruvate and D,L-GAP, and IspC (DXP reductoisomerase) as a coupling enzyme as reported previously.[7] Under these conditions D-GAP from a solution of D,L-GAP reacts exclusively to form DXP.[10] Here we have used commercially available racemic D,L-GAP by knowing that L-GAP would not affect DXP synthase activity.[1,5,6] The concentration of D-GAP was calculated as one-half of the D,L-GAP concentration.

CD Spectroscopy. CD spectra were recorded on a Applied Photophysics Chirascan CD spectrometer (Leatherhead, U.K.) in 2.4 mL volume with 1 cm path length cell.

Formation of LThDP from Pyruvate by DXP Synthase. DXP synthase (55.9 μM active centers) was titrated with pyruvate (0.05−1 mM) in 100 mM HEPES (pH 8.0) containing 100 mM NaCl, 0.2 mM ThDP, and 1 mM $MgCl_2$ at 4 °C. After apparent saturation with 1 mM pyruvate, 250 μM D-GAP was added. The K_d,app was calculated by fitting the data to a Hill function (equation S1, see Supporting Information).

Stopped-Flow CD Spectroscopy. Kinetic traces were recorded on a Pi*-180 stopped-flow CD spectrometer (Applied Photophysics, U.K.) using 10 mm path length. ThDP-bound intermediates (LThDP specifically) were detected at 313 nm and DXP at 297 nm, as dictated by the high sensitivity of the lamp at those wavelengths. Data from 10 repetitive shots were averaged and fit to the appropriate equation using Sigma Plot v.10.0.

$$CD_\lambda(t) = CD_1 e^{-k_1 t} + c \quad (1)$$

$$CD_\lambda(t) = CD_1 e^{-k_1 t} + CD_2 e^{-k_2 t} - CD_3 e^{-k_3 t} + c \quad (2)$$

$$CD_\lambda(t) = CD_1 e^{-k_1 t} - CD_3 e^{-k_3 t} + c \quad (3)$$

$$CD_\lambda(t) = CD_1 e^{-k_1 t} + CD_2 e^{-k_2 t} + c \quad (4)$$

where k_1, k_2, and k_3 are the apparent rate constants, c is $CD^{max}{}_\lambda$ in the exponential rise to maximum model or $CD^{min}{}_\lambda$ in the exponential decay model.

Presteady State Formation of 1′,4′-IminoLThDP. DXP synthase (68.1 μM active centers) in one syringe was mixed with equal volume of 2 mM pyruvate in the second syringe under presteady state conditions in buffer A (50 mM Tris (pH 8.0), 100 mM NaCl, 0.5 mM ThDP, 2.0 mM $MgCl_2$, 1 mM DTT, 1% glycerol) at 6 °C. The reaction was monitored at 313 nm for 7 s. Data were fit to a single-exponential model as in eq 1.

Single Turnover Experiments of DXP Synthase with Pyruvate in the Absence of D-GAP. DXP synthase (81.4 μM active centers) in one syringe was mixed with equal volume of pyruvate (50 μM) in the second syringe, both in buffer A at 6 °C. The reaction was monitored at 313 nm for 50 s, and data were fit to a triple exponential model as in eq 2. The same experiment was carried out at 37 °C, and data were fit to double exponential model as in eq 3.

Presteady State Decarboxylation of 1′,4′-IminoLThDP in the Presence of D-GAP. DXP synthase (68.1 μM active centers) was premixed with 500 μM pyruvate at 6 °C to form LThDP in one syringe, then rapidly mixed with an equal volume of 100 μM D-GAP in the second syringe, both in buffer A at 6 °C. A similar experiment was performed using D-GAP (0.05−0.25 mM), and data were fit to eq 1.

Presteady State Formation of DXP. DXP synthase (1.04 μM active centers) in buffer A placed in one syringe was mixed with an equal volume of 4 mM pyruvate and 2 mM D-GAP in the same buffer placed in the second syringe. Reaction was monitored by CD over a period of 100 s at 6 °C at 297 nm, and data were fitted to a double-exponential model as in eq 4.

NMR Spectroscopy. NMR spectra were acquired on a Varian INOVA 600 MHz instrument. The water signal was suppressed by presaturation.

Detection of LThDP by ^{1}H NMR Spectroscopy. The reaction mixture containing DXP synthase (459.3 μM active centers) in 20 mM Tris (pH 8.0), 100 mM NaCl, 0.1 mM ThDP, and 0.5 mM $MgCl_2$ was mixed with 1 mM pyruvate in the same buffer. The reaction mixture was incubated at 5 °C for 5 s (and an identical sample for 20 s) and quenched with 12.5% TCA in 1 M DCl/D_2O. The mixture was centrifuged at 15 700 g for 20 min, and the ^{1}H NMR spectrum of the supernatant was recorded using 16 384 scans with a recycle delay of 2.0 s.

Detection of DXP Product by ^{1}H NMR Spectroscopy. A reaction mixture containing DXP synthase (22.22 μM active centers) in 20 mM KH_2PO_4 (pH 7.5) containing 100 mM NaCl, 0.1 mM ThDP, 0.5 mM $MgCl_2$, and 1 mM DTT was mixed with 15 mM D-GAP and 10 mM pyruvate in the same buffer. After overnight incubation at 4 °C, the ^{1}H NMR spectrum of the supernatant was recorded.

dx.doi.org/10.1021/ja307315u | J. Am. Chem. Soc. 2012, 134, 18374−18379

RESULTS

The Tautomeric and Ionization States of ThDP on DXP Synthase. The near-UV CD spectrum of DXP synthase displays a negative band at 320 nm (Figure S1A of the Supporting Information), suggesting the preponderance of the AP tautomer of ThDP bound to the enzyme.[9] Next, the CD spectrum was recorded at different pH values to convert the AP form of ThDP to the APH^+ form (Scheme 1, left). Reducing the pH from 7.98 to 7.07 gradually diminished the amplitude of the CD band at 320 nm (Figure S1A of the Supporting Information), indicating that the AP form of ThDP is dominant at pH 7.98, and the APH^+ form is dominant at pH 7.07 [while the APH^+ form has no CD signature identified so far, its existence on three ThDP enzymes was recently demonstrated by solid-state NMR].[11] A plot of the CD320 against pH could be fitted to a single proton titrating with a pK_a of 7.5 (Figure S1A of the Supporting Information, inset) for the protonic equilibrium ([AP] + [IP])/[APH^+]. This pK_a elevation compared to the value of 4.85 in water[12] has been attributed to interaction via the short hydrogen bond between the N1′-atom of the 4′-aminopyrimidine and the highly conserved glutamate side chain.

Next, we determined the pH dependence of the rate of formation of DXP by DXP synthase to establish whether the pK_a of the APH^+ bears any relationship to the pH dependence of the activity. The rate of formation of DXP by DXP synthase determined at pH values of 6.65−8.08 (Figure S1B of the Supporting Information) revealed a pK_{app} of 7.6 (Figure S1B of the Supporting Information inset), which correlates well with the pK_a of 7.5 for the protonic equilibrium ([AP]+[IP])/[APH^+] (Figure S1A of the Supporting Information, inset). That is the pK_a of the enzyme-bound APH^+ form is very near the pH of optimal activity of the enzyme[7] signaling the need for all forms APH^+, AP, and IP in the mechanism, in accord with results on five other ThDP enzymes, thus creating a general trend.[13]

Direct Observation of the Predecarboxylation LThDP Intermediate on DXP Synthase. We next turned to CD studies of DXP synthase with the donor substrate pyruvate. On addition of saturating pyruvate at 4 °C, there developed a positive CD band at 313 nm (Figure 1, left), gradually replacing

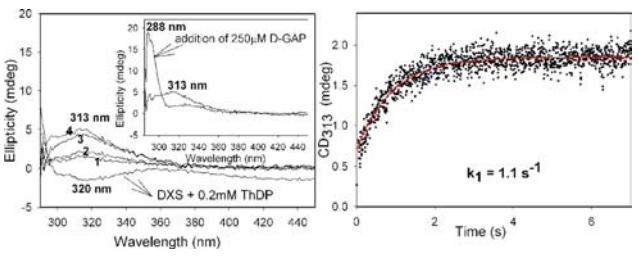


Figure 1. Formation of 1′,4′-iminoLThDP from pyruvate by DXP synthase. Left: CD titration of DXP synthase with pyruvate (1) 50 μM, (2) 100 μM, (3) 150 μM, (4) 1 mM. Inset: addition of 250 μM D-GAP generated DXP. Right; rate of 1′,4′-iminoLThDP formation at 6 °C.

the negative CD band at 320 nm assigned to the AP form of ThDP (Scheme 1). The CD band at 313 nm revealed apparent saturation with pyruvate (apparent $K_{d,pyruvate}$ ~90 μM, Figure S2 of the Supporting Information) and was tentatively assigned to the 1′,4′-iminopyrimidinylLThDP. A stopped-flow CD experiment was designed to determine the rate of interconversion of the ThDP-bound covalent intermediates on DXP synthase. In the presteady state experiment, DXP synthase (68.1 μM active centers) in one syringe was rapidly mixed with saturating concentration of pyruvate (2 mM) in the second syringe, giving rise to an increase at 313 nm and reaching a steady state in 2 s with rate constant of 1.1 ± 0.03 s^{-1} (Figure 1, right).

The species represented by the positive CD band at 313 nm (signifying the presence of the IP tautomeric form of ThDP) could be assigned as either the predecarboxylation intermediate (LThDP) or the postdecarboxylation ThDP-bound DXP intermediate (Scheme 1, right), since both carry tetrahedral substitution at C2α. To resolve the ambiguity, an NMR method was used,[14] a method that recognized that the chemical shift of the C6′-H resonance is different for LThDP (7.26 ppm)[14] and C2α- hydroxyethylThDP (HEThDP, 7.33 ppm)[14] and indeed for carboligated product-ThDP adducts as well. Accordingly, an NMR spectrum of a mixture of DXP synthase with pyruvate quenched in 5 or 20 s revealed a chemical shift of 7.26 ppm, confirming that the CD at 313 nm corresponds to LThDP (Figure S3 of the Supporting Information).[14] Integration of the C2-H and C6′-H resonances showed that approximately the same percent of ThDP was converted to LThDP in 5 s (~50%) and in 20 s (~55%), thus confirming the remarkable stability of LThDP on DXP synthase.

To interpret data once both pyruvate and D-GAP are present, we need to first establish rates of LThDP formation and decarboxylation in the absence of D-GAP. We accomplished this by a single turnover stopped-flow CD experiment with DXP synthase concentration in excess of pyruvate, carried out at 6 and 37 °C under the same conditions, the latter to enable comparison with alternative fates of the enamine intermediate. DXP synthase (81.4 μM active centers) in one syringe was mixed with an equal volume of pyruvate (50 μM) in the second syringe. The rates of LThDP formation and decarboxylation are k_1 = 1.4 ± 0.05 s^{-1} and k_2 = (7 ± 0.01) × 10^{-2} s^{-1} at 6 °C (Figure 2A) and k_1 = 2.8 ± 0.15 s^{-1} and k_2 = (4.9 ± 0.01) × 10^{-1} s^{-1} at 37 °C (Figure 2B, Table 1).

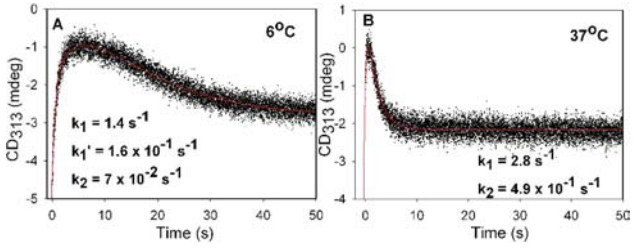


Figure 2. Formation and decarboxylation of LThDP on DXP synthase in the absence of D-GAP under single turnover conditions (A) at 6 °C and (B) at 37 °C.

Table 1. Rate Constants for LThDP Formation and Decarboxylation on DXP Synthase under Presteady State Conditions[a]

reaction with DXP synthase	k_1 (s^{-1})	k_2 (s^{-1})
pyruvate, 6 °C	1.1 ± 0.03	
pyruvate (single turnover, 6 °C)	1.4 ± 0.05	(7 ± 0.01) × 10^{-2}
pyruvate (single turnover, 37 °C)	2.8 ± 0.15	(4.9 ± 0.01) × 10^{-1}
pyruvate + D-GAP, 6 °C	(6.8 ± 0.01) × 10^{-1}	(4.2 ± 1.0) × 10^{1}

[a]See detailed conditions in Experimental section; see Scheme 1 right for assignment of rate constants to individual steps.

dx.doi.org/10.1021/ja307315u | J. Am. Chem. Soc. 2012, 134, 18374−18379

Direct Observation of the Rate of LThDP Decarboxylation in the Presence of D-GAP. Next, we wished to determine the effect of the acceptor substrate D-GAP on the rate of decarboxylation of LThDP. For these experiments, commercially available D,L-GAP was used as the source of D-GAP; L-GAP does not act as substrate for the enzyme (Figure S6 of the Supporting Information).[1,5,6,10] Addition of 250 μM D-GAP to DXP synthase with preformed LThDP (displayed a positive CD band at 313 nm in Figure 1, inset) resulted in formation of a new positive band at 288 nm, replacing the 313-nm band (Figure 1 left, inset). The CD spectrum of the supernatant after protein was removed indicated the persistence of the band at 288 nm, suggesting that it corresponds to the DXP product. Confirmation of the product was obtained with an NMR sample, prepared using DXP synthase (22.2 μM active centers) with pyruvate (10 mM) and D-GAP (15 mM). The ^{1}H NMR spectrum identified the DXP in the supernatant by comparison with the spectrum of an "authentic" commercially available DXP sample and that reported.[1]

Having observed accumulation of LThDP on the enzyme (Figure 1, left), we reasoned that direct monitoring of the CD at 313 nm (corresponding to LThDP) would enable us to directly measure the rate of decarboxylation upon the addition of D-GAP. As seen in Figure 1 left inset, the decarboxylation of LThDP is accelerated in the presence of the acceptor substrate D-GAP. To obtain quantitative support for this hypothesis, LThDP was preformed in one syringe by mixing DXP synthase (68.1 μM active centers) and pyruvate (500 μM) at 6 °C and then was rapidly mixed with D-GAP (100 μM) placed in the second syringe on the stopped-flow CD instrument. Time-dependent decarboxylation of LThDP (CD at 313 nm, Figure 3A) was observed with a rate constant of (3.0 ± 0.9) × 10^1 s^{-1}

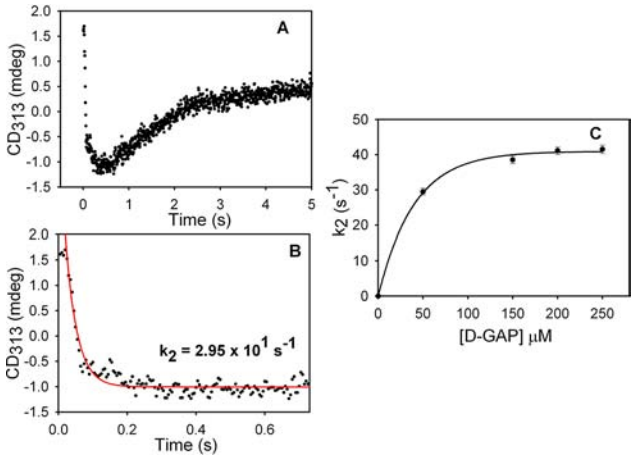


Figure 3. (A) Time course of LThDP decarboxylation and resynthesis by DXP synthase in the presence of D-GAP at 6 °C. (B) Expansion of early behavior in A fitting data to eq 1. (C) Dependence of the rate of LThDP decarboxylation on final concentration of D-GAP.

(Figure 3B), a rate constant that increases to (4.2 ± 1) × 10^1 s^{-1} for DXP synthase saturated with D-GAP (Figure 3C). An interpretation of the total behavior at 313 nm (Figure 3A) is that decarboxylation proceeds until all of the D-GAP is consumed (drop in the curve; the concentration of D-GAP reacting is 50 μM after mixing), after which time the remaining pyruvate forms more LThDP.

To determine the rate of DXP release by CD, DXP synthase (1.04 μM active centers) in one syringe was mixed with pyruvate (4 mM) and D-GAP (2 mM) in the second syringe at 6 °C, providing a rate constant k_4 = 1.2 ± 0.03 s^{-1} (Figure S4 of the Supporting Information, bottom). The lower concentration of DXP synthase assured that the CD observations pertain to formation of free, rather than enzyme-bound DXP.

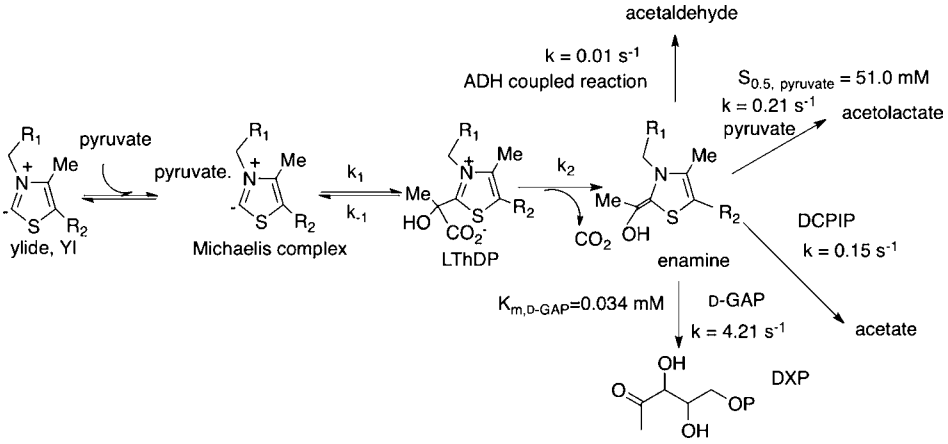
Putative Acceptors for Reactions of the Enamine. As shown in Scheme 2, one could envision alternative competing trapping of the enamine resulting from LThDP decarboxylation, and the rates of these reactions needed to be established under the reaction conditions. A CD method was developed to measure the steady-state kinetic parameters for DXP synthase providing data consistent with the previous coupled assay.[7] (1) With D-GAP as acceptor, the rate of DXP formation (4.21 s^{-1}) at 37 °C was calculated from the slope of the progress curves recorded by CD with time at 290 nm. To calculate the rate constant, the molar ellipticity of DXP (13 200 deg cm^2 dmol^{-1}) was determined (enzyme assays shown in the Supporting Information). (2) With a proton electrophile the enamine would be converted to HEThDP followed by release of acetaldehyde, whose rate of formation with the YADH/NADH coupled assay was 0.01 s^{-1}. (3) With pyruvate as acceptor, carboligation of the enamine leads to (*R*)-acetolactate (negative CD band at 300 nm) in accord with the findings by the Johns Hopkins group.[10] A CD titration of DXP synthase with pyruvate (1−90 mM) revealed a negative band centered at 300 nm, and the CD amplitude at 300 nm increased with increasing pyruvate concentration (Figure S5 of the Supporting Information). Plotting the CD ellipticity at 300 nm against [pyruvate] gave $S_{0.5}$ for pyruvate of 51.0 ± 1.7 mM (Figure S5 of the Supporting Information inset), and a k_{cat} = 0.21 s^{-1} was calculated. (4) Finally, the DCPIP reduction assay was carried out as evidence of decarboxylation, giving a rate constant of 0.15 s^{-1}.

The rates of product formation from these putative acceptors at 37 °C (Scheme 2) indicate that formation of DXP is the preferred outcome by a significant factor but that DXP synthase could indeed also produce significant (*R*)-acetolactate under some conditions.

DISCUSSION

Perhaps the most significant observation made from steady state CD was of a "stable" ThDP-bound predecarboxylation intermediate with tetrahedral C2α substitution at 4 °C on DXP synthase in the absence of D-GAP; confirmed by NMR to be LThDP. Stopped-flow CD experiments enabled us to measure the rates of key steps on the putative reaction pathway of DXP synthase at 6 °C: the rate constant for formation of LThDP (1.1 s^{-1}, k_1 in Scheme 1), the rate constant of decarboxylation (4.2 × 10^1 s^{-1}, k_2 in Scheme 1 in the presence of D-GAP and 7 × 10^{-2} s^{-1} in its absence), the rate constant for carboligation k_3 remains unknown but is almost certainly not rate limiting, and the rate constant for DXP release (1.2 s^{-1}, k_4 in Scheme 1). On the basis of this information, LThDP formation appears to be rate limiting. Most notable is the sizable rate acceleration on the decarboxylation step caused by the acceptor substrate D-GAP (Scheme 1). We note that in accord with our findings, Eubanks and Poulter[4] first proposed a unique requirement for ternary complex formation in DXP synthase catalysis on the basis of CO_2 trapping studies that showed significant reduction in rate of CO_2 release in the absence of D-GAP. That study suggested that the presence of D-GAP is required for CO_2 release; however, until now it was unknown whether binding of D-GAP facilitated formation of LThDP when both substrates are

Scheme 2. Putative Acceptors for the Enamine Reactions under Steady State Conditions at 37 °C



bound, or promoted decarboxylation of the LThDP intermediate. The CD approach used here permits the interrogation of these individual steps. Here, we show that the LThDP intermediate readily forms upon binding of pyruvate to DXP synthase in the absence of D-GAP, and persists at low temperature until addition of D-GAP.

The collective reports on DXP synthase mechanism are conflicting. Eubanks and Poulter[4] provided the earliest compelling evidence for the requirement of a ternary complex and proposed an ordered mechanism with pyruvate binding first. More recently, reports by Matsue et al.[6] and Brammer et al.[7] have proposed ping-pong or random sequential mechanisms, respectively. On the basis of the evidence here that the LThDP intermediate persists at low temperature until addition of D-GAP, a classical ping-pong mechanism seems unlikely. The present results also exclude an ordered mechanism requiring GAP binding in the first step, since LThDP formation can clearly take place upon binding of pyruvate to free enzyme. The ordered and random sequential mechanisms previously proposed[4,10] both support the idea that ternary complex formation is uniquely required in DXP synthase catalysis, in accordance with our findings here. A random substrate binding model cannot be directly tested here, as the presence of GAP facilitates rapid decarboxylation and precludes build-up of LThDP. While these results could support an ordered mechanism where pyruvate binds first, this CD analysis does not exclude the possibility that pyruvate can bind to an *E*-D-GAP complex and undergo conversion to LThDP in the presence of GAP, prior to GAP-promoted decarboxylation.

The 600-fold increase in rate of decarboxylation by DXP synthase as a result of D-GAP binding appears to be unique and supports the idea that ternary complex formation is required. The results should be evaluated in view of relevant studies on the decarboxylation of enzyme-bound LThDP. Tittmann and Wille summarized data on the ability to observe LThDP on a variety of ThDP enzymes[15] and concluded the "LThDP to be extremely short lived and marginally accumulated at steady state, rendering a structural characterization of LThDP under steady state turnover or single turnover conditions almost impossible;" although the NMR method did detect some LThDP in certain cases, such as in the F479W pyruvate oxidase variant.[26] These observations were also summarized in a review by Kluger and Tittmann.[16] In the experience of the Rutgers group, turnover numbers for ThDP-dependent pyruvate decarboxylating enzymes are in the range of $(5-6) \times 10^1$ s^{-1}, hence the rate constant for decarboxylation of enzyme-bound LThDP must be at least this large, more in the range of the rate constant here observed with D-GAP present.

Taking into consideration the long history of studies on rates of decarboxylation using nonenzymatic LThDP models, it could be inferred that the dielectric constant of the putative DXP synthase active site of the ternary complex may be low. Lienhard and co-workers showed that transferring the 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethyl-thiazolium ion from water to ethanol led to a 10^4-10^5 fold increase in the decarboxylation rate.[17] Kluger et al. reported a rate constant of 4×10^{-5} s^{-1} for decarboxylation of a C2α-lactylthiamin[18] at pH 7.0 and 25 °C. More recently, a study by Zhang et al. demonstrated "spontaneous" decarboxylation of C2α-lactylthiazolium salts with rate constants exceeding 50 s^{-1} in THF, simply by decreasing the dielectric constant of the solvent.[19] Evidence supporting the idea of an apolar active site of yeast pyruvate decarboxylase (YPDC) was reported by Jordan et al.[20] in a study utilizing thiochrome pyrophosphate, a fluorescent ThDP analog whose emission spectra correlates to solvent polarity. The effective active site dielectric constant for YPDC was interpolated to that between 1-hexanol and 1-pentnol (i.e., between 11 and 13), despite the existence of a Glu, an Asp, and two His residues (in addition to the conserved glutamate) at the active center.

In view of these nonenzymatic models for LThDP decarboxylation rates reported in the previous paragraph, apparently, DXP synthase accelerates the rate of decarboxylation [$k_2 = (7 \pm 0.01) \times 10^{-2}$ s^{-1} at 6 °C] compared to the value reported in aqueous buffer (4×10^{-5} s^{-1}). Yet, the lifetime of LThDP on DXP synthase in the absence of D-GAP is still surprisingly long in comparison with other enzymes carrying out the same function (see above). The 600-fold rate acceleration on addition of D-GAP on DXP synthase translates to an energy barrier lowering of 3.5 kcal/mol, achieving a rate constant very similar to that achieved in some nonenzymatic model systems.

The slow rate of decarboxylation of LThDP on the enzyme in the absence of D-GAP requires explanation.

A likely explanation to consider for the effect of D-GAP is that decarboxylation is reversible[21−23] and CO_2 release from the enzyme is potentiated by D-GAP binding. Several reports by Kluger et al. have focused on model studies of decarboxylation that have led to the proposal that decarboxylation is reversible on the ThDP enzyme benzoylformate decarboxylase.[16,21] For

dx.doi.org/10.1021/ja307315u | J. Am. Chem. Soc. 2012, 134, 18374−18379

benzoylformate decarboxylase and A28S benzaldehyde lyase, it was suggested that serine residue from the active site may act as nucleophile in CO_2 trapping.[24] The fact that we only observed LThDP both on the enzyme and that once it is released from the enzyme suggests that if the LThDP is indeed at equilibrium with the enamine + CO_2, the acid quench should produce HEThDP. Given that HEThDP is not observed does not argue against this scenario, but suggests that the equilibrium lies far to the LThDP side.

A different explanation for the effect of D-GAP on the decarboxylation rate is based upon our understanding of the decarboxylation mechanism provided by Lienhard's work[25] and the need for a zwitterion charge distribution (positively charged thiazolium ring with carboxylate ionization state of the lactyl group) for optimal decarboxylation rate. Thus, it is possible that protonation of the carboxylate group by DXP synthase active site residues could reduce the rate of decarboxylation.

Understanding the origins of the slow decarboxylation rate in the absence of D-GAP and its acceleration in its presence could create a new opportunity for the design of selective inhibitors against this important enzyme.

CONCLUSION

In summary, a combination of steady state and time-resolved (presteady state) CD spectroscopy were used to study the individual rate constants in the DXP synthase reaction (Table 1 and Scheme 1). The results clearly demonstrate that formation of LThDP is the rate-limiting step. Apparently, the acceptor substrate D-GAP accelerates decarboxylation of LThDP significantly, while in the absence of D-GAP, decarboxylation is very slow. It is also clear that, among the putative acceptors for the reaction with the enamine, D-GAP is the preferred acceptor.

ASSOCIATED CONTENT

Supporting Information

Experimental conditions for DXP synthase enzyme assays and pH dependence of AP form of ThDP on DXP synthase and DXP formation. Figures representing pH dependence, Michaelis–Menten plot to determine the K_m for D-GAP, HPLC analysis demonstrating utilization of D-GAP only from a racemic mixture, and NMR spectra of DXP product. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

cmeyers@jhmi.edu; frjordan@rutgers.edu

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Supported at JHU by NIH-GM084998 and at Rutgers by NIH-GM-050380. We gratefully acknowledge Jessica Smith and Katie Heflin for assistance with the purification and activity determination of wild-type DXP synthase used in this study.

REFERENCES

(1) Sprenger, G. A.; Schörken, U.; Wiegert, T.; Grolle, S.; de Graaf, A. A.; Taylor, S. V.; Begley, T. P.; Bringer-Meyer, S.; Sahm, H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12857–12862.
(2) Lois, L. M.; Campos, N.; Putra, S. R.; Danielsen, K.; Rohmer, M.; Boronat, A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2105–2110.
(3) Hill, R. E.; Himmeldirk, K.; Kennedy, I. A.; Pauloski, R. M.; Sayer, B. G.; Wolf, E.; Spenser, I. D. *J. Biol. Chem.* **1996**, *271*, 30426–30435.
(4) Eubanks, L. M.; Poulter, C. D. *Biochemistry* **2003**, *42*, 1140–1149.
(5) Sisquella, X.; de Pourcq, K.; Alguacil, J.; Robles, J.; Sanz, F.; Anselmetti, D.; Imperial, S.; Fernàndez-Busquets, X. *FASEB J.* **2010**, *24*, 4203–4217.
(6) Matsue, Y.; Mizuno, H.; Tomita, T.; Asami, T.; Nishiyama, M.; Kuzuyama, T. *J. Antibiot.* **2010**, *63*, 583–588.
(7) Brammer, L. A.; Smith, J. M.; Wade, H.; Meyers, C. F. *J. Biol. Chem.* **2011**, *286*, 36522–36531.
(8) Nemeria, N.; Chakraborty, S.; Baykal, A.; Korotchkina, L. G.; Patel, M. S.; Jordan, F. *Proc. Natl. Acad. Sci. USA.* **2007**, *104*, 78–82.
(9) Nemeria, N. S.; Chakraborty, S.; Balakrishnan, A.; Jordan, F. *FEBS J.* **2009**, *276*, 2432–2446.
(10) Brammer, L. A.; Meyers, C. F. *Org. Lett.* **2009**, *11*, 4748–4751.
(11) Balakrishnan, A.; Paramasivam, S.; Chakraborty, S.; Pole nova, T.; Jordan, F. *J. Am. Chem. Soc.* **2012**, *134*, 665–672.
(12) Cain, A. H.; Sullivan, G. R.; Roberts, J. D. *J. Am. Chem. Soc.* **1977**, *99*, 6423–6425.
(13) Nemeria, N.; Korotchkina, L.; McLeish, M. J.; Kenyon, G. L.; Patel, M. S.; Jordan, F. *Biochemistry* **2007**, *46*, 10739–10744.
(14) Tittmann, K.; Golbik, R.; Uhlemann, K.; Khailova, L.; Schneider, G.; Patel, M.; Jordan, F.; Chipman, D. M.; Duggl by, R. G.; Hübner, G. *Biochemistry* **2003**, *42*, 7885–7891.
(15) Tittmann, K.; Wille, G. *J. Mol. Catal. B* **2009**, *61*, 93–99.
(16) Kluger, R.; Tittmann, K. *Chem. Rev.* **2008**, *108*, 1797–1833.
(17) Crosby, J.; Lienhard, G. E. *J. Am. Chem. Soc.* **1970**, *92*, 5707–5716.
(18) Kluger, R.; Chin, J.; Smyth, T. *J. Am. Chem. Soc.* **1981**, *103*, 884–888.
(19) Zhang, S.; Liu, M.; Yan, Y.; Zhang, Z.; Jordan, F. *J. Biol. Chem.* **2004**, *279*, 54312–54318.
(20) Jordan, F.; Li, H.; Brown, A. *Biochemistry* **1999**, *38*, 6369–6373.
(21) Mundle, S. O. C.; Rathgeber, S.; Lacrampe-Couloume, G.; Sherwood Lollar, B.; Kluger, R. *J. Am. Chem. Soc.* **2009**, *131*, 11638–11639.
(22) Häussermann, A.; Rominger, F.; Straub, B. F. *Chem.–Eur. J.* **2012**, DOI: 10.1002/chem..201202298.
(23) Gonzalez-James, O. M.; Singleton, D. A. *J. Am. Chem. Soc.* **2010**, *132*, 6896–6897.
(24) Brandt, G. S.; Kneen, M. M.; Petsko, G. A.; Ringe, D.; McLeish, M. J. *J. Am. Chem. Soc.* **2010**, *132*, 438–439.
(25) Crosby, J.; Stone, R.; Lienhard, G. E. *J. Am. Chem. Soc.* **1970**, *92*, 2891–2900.
(26) Wille, G.; Meyer, D.; Steinmetz, A.; Hinze, E.; Golbik, R.; Tittmann, K. *Nat. Chem. Biol.* **2006**, *2*, 324–328.