Mechanism of Benzaldehyde Lyase Studied via Thiamin Diphosphate-Bound Intermediates and Kinetic Isotope Effects[†]

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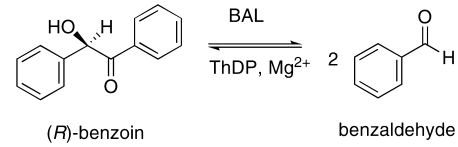
ABSTRACT: Direct spectroscopic observation of thiamin diphosphate-bound intermediates was achieved on the enzyme benzaldehyde lyase, which carries out reversible and highly enantiospecific conversion of (*R*)-benzoin to benzaldehyde. The key enamine intermediate could be observed at λ_{max} 393 nm in the benzoin breakdown direction and in the decarboxylase reaction starting with benzoylformate. With benzaldehyde as substrate, no intermediates could be detected, only formation of benzoin at 314 nm. To probe the rate-limiting step in the direction of (*R*)-benzoin synthesis, the $^1H/^2H$ kinetic isotope effect was determined for benzaldehyde labeled at the aldehyde position and found to be small (1.14 ± 0.03), indicating that ionization of the C2αH from C2α-hydroxybenzylthiamin diphosphate is not rate limiting. Use of the alternate substrates benzoylformic and phenylpyruvic acids (motivated by the observation that while a carboligase, benzaldehyde lyase could also catalyze the slow decarboxylation of 2-oxo acids) enabled the observation of the substrate-thiamin covalent intermediate via the 1′,4′-iminopyrimidine tautomer, characteristic of all intermediates with a tetrahedral C2 substituent on ThDP. The reaction of benzaldehyde lyase with the chromophoric substrate analogue (*E*)-2-oxo-4(pyridin-3-yl)-3-butenoic acid and its decarboxylated product (*E*)-3-(pyridine-3-yl)acrylaldehyde enabled the detection of covalent adducts with both. Neither adduct underwent further reaction. An important finding of the studies is that *all* thiamin-related intermediates are in a chiral environment on benzaldehyde lyase as reflected by their circular dichroism signatures.

The enzyme benzaldehyde lyase from the strain *Pseudomonous fluorescens* Biovar I (BAL; EC 4.1.2.38) is a relatively recently discovered thiamin diphosphate (ThDP[1])-dependent enzyme, which catalyzes the reversible conversion of (*R*)-benzoin to two molecules of benzaldehyde ((*1*) eq 1). The enzyme is a valuable tool in chemoenzymatic synthesis of α-hydroxyketones (*2*). The proposed mechanism for the reversible benzoin condensation catalyzed by BAL (Scheme 1) starts with a nucleophilic attack at the carbonyl carbon of (*R*)-benzoin by the ThDP ylide producing the first covalent intermediate C2-(α,β-dihydroxy-α,β-diphenyl)-ethyl ThDP (DDEThDP). The DDEThDP releases the first molecule of benzaldehyde producing the enamine, which undergoes C2α-protonation to form C2α-hydroxybenzylThDP (HBThDP). A second molecule of benzaldehyde is then released, and the ThDP ylide is regenerated (Scheme 1).



(1)

The crystal structure of BAL (*3*) confirmed that the enzyme is a homotetramer of 4 × 563 amino acid residues with a molecular mass of 58,919 Da/monomer, reminiscent in size to other ThDP-dependent 2-oxo acid decarboxylases (*2, 4–6*). Each subunit binds one ThDP molecule using one Mg^{2+} ion as a scaffold for the diphosphate moiety. BAL shares common structural features with most other ThDP enzymes: (a) a glutamate residue (E50) within hydrogen bonding distance from N1′ of ThDP; (b) the ThDP exists in the unusual "V" coenzyme conformation, which brings the N4′ atom of the 4′-aminopyrimidine and the C2 thiazolium atom to within 3.5 Å of each other. A particularly unusual feature of BAL from a mechanistic viewpoint is that, with the exception of E50 and a histidine residue (H29), the active center is devoid of any acid–base amino acid residues (Figure 1).

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[1] Abbreviations: BF, benzoylformate; ThDP, thiamin diphosphate; MThDP, C2α-mandelylThDP, the covalent adduct formed between ThDP and benzoylformate; HBThDP, C2α-hydroxybenzylThDP, the covalent adduct formed between ThDP and the product of the reaction benzaldehyde; BFDC, benzoylformate decarboxylase; 3-PKB, (*E*)-2-oxo-4(pyridin-3-yl)-3-butenoic acid; MThDP*, the predecarboxylation intermediate analogue formed between ThDP and 3-PKB; PAA, *(E)*-3-(pyridine-3-yl)acrylaldehyde; DDEThDP, C2-(α,β-dihydroxy-α,β-diphenyl)ethyl-ThDP; PDA, photodiode array; SVD, single value decomposition; AP, the 4′-aminopyrimidine tautomeric form of ThDP and derivatives; APH^+, the N1′-protonated 4′-aminopyrimidinium form of ThDP and derivatives; IP, the 1′,4′-iminopyrimidine tautomeric form of ThDP and derivatives.

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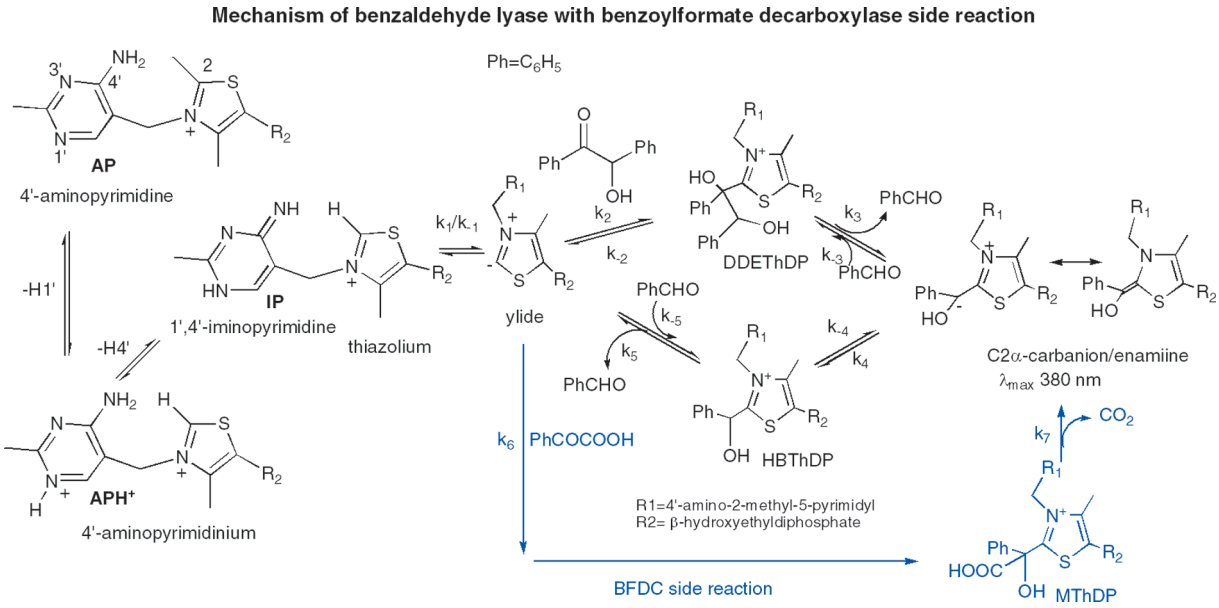
Scheme 1: Proposed Mechanism of Reversible Benzoin Condensation in Benzaldehyde Lyase[a]



[a] The benzoylformate decarboxylase side reaction is shown in blue.

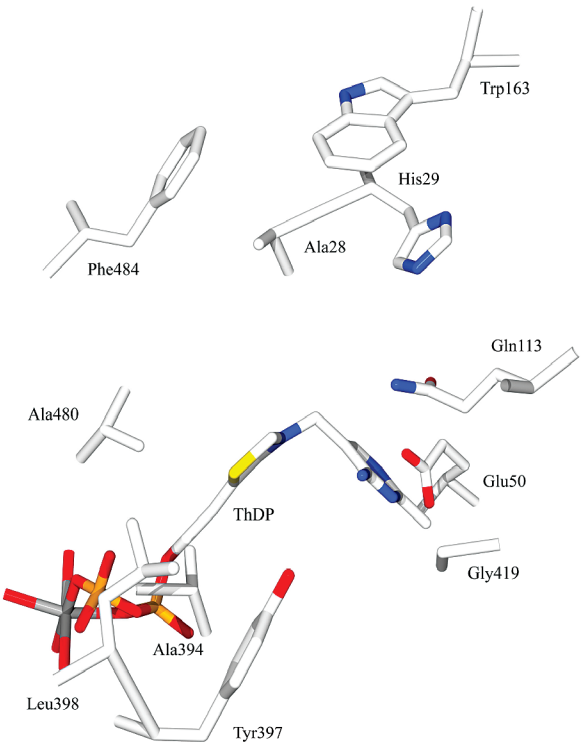


FIGURE 1: Active site of benzaldehyde lyase. The active site image was generated from the PDB file 2AG0 [*3*] using Swiss-PdbViewer (www.expasy.org/spdbv) (*28*) and POV-Ray (www.povray.org).

While widely used for asymmetric syntheses of various α-hydroxyketones, relatively little has been reported on the mechanism of catalysis of BAL. Direct observation, characterization of ThDP-bound intermediates and determination of individual rate constants for their interconversion in the catalytic cycle of BAL remain challenging.

We have developed spectroscopic tools, including chromophoric alternate substrates, to obtain evidence regarding the mechanism of ThDP-dependent enzymes. These have been used for the detection of ThDP-bound intermediates on yeast pyruvate decarboxylase (YPDC; *9–11*) and benzoylformate decarboxylase (BFDC; (*11*)), enzymes which share some aspects of mechanism with BAL. While a lyase/carboligase in its principal function, BAL does catalyze slow decarboxylation of some aromatic 2-oxo acids, and it was thought that this property could be utilized to facilitate the observation of ThDP-bound intermediates. The objective of this study is to characterize ThDP-bound intermediates on BAL and their time-course of formation/depletion using both the substrate/product and analogues, and to obtain further information regarding the state of tautomerization of ThDP and ThDP-bound intermediates on the reaction pathway.

Here we report studies with benzoylformic and phenylpyruvic acids, as well as the chromophoric analogue, (*E*)-2-oxo-4-(pyridin-3-yl)-but-3-enoic acid (3-PKB), previously used for YPDC and BFDC (*9–11*). Each of these compounds adds to our understanding of the mechanism of BAL since they enable us to detect all of the ThDP-bound intermediates, as well as their chiral behavior on the enzyme. In addition, we have established the state of ionization and tautomeric nature of the 4′-aminopyrimidine ring of ThDP on BAL, an ongoing focus of our work at Rutgers and in this collaborative research (*5, 6, 12–19*). We have now demonstrated in a number of ThDP-dependent enzymes that the 4′-aminopyrimidine (AP) ring of ThDP actively participates and plays a very crucial role in catalysis via its 1′,4′-iminopyrimidine tautomeric (IP) form. The most salient conclusion from those studies, a conclusion that we believe is applicable to all ThDP enzymes, is that, in the catalytic cycle, any covalent ThDP-bound intermediate with four substituents at the C2α atom exists in the 1′,4′-iminopyrimidine tautomeric form. Finally, we have probed the rate-limiting step of BAL in the direction of benzoin formation.

EXPERIMENTAL PROCEDURES

Materials and Methods. Benzoylformic acid, benzaldehyde, phenylpyruvic acid, (*E*)-3-(pyridine-3-yl)acrylaldehyde were from Sigma-Aldrich (St. Louis, MO). [7-^{2}H]-benzal-

dehyde was from Cambridge Isotope Laboratories, MA. The 3-PKB was synthesized as reported elsewhere (*9*).

Expression and Purification of BAL. The plasmid pKK-BAL-His for expression of wild-type BAL was transformed into BL21(DE3)pLysS cells (Novagen). Expression and purification were carried out essentially as described in (*20*). The fractions of highest purity as assessed by SDS−PAGE were pooled, and the buffer was exchanged for the storage buffer (50 mM potassium phosphate buffer at pH 6.5, 1 mM $MgSO_4$, 0.5 mM ThDP, 10% glycerol) using Econo-Pac 10 DG desalting columns (BioRad). The protein samples were concentrated with Amicon Ultra centrifugal filters (Millipore) and stored at −80 °C. The enzyme preparations were apparently homogeneous as judged by SDS−PAGE. Protein concentration was determined with the Bradford assay (*21*) using bovine serum albumin as standard.

Activity and Related Measurements. The activity of BAL was measured in 1 mL of 50 mM Tris-HCl buffer (pH 8.0), containing 1 mg/mL of bovine serum albumin, 1 mM $MgSO_4$, 0.50 mM ThDP, 0.25 units of horse liver alcohol dehydrogenase (HLADH; 12.5 units/mL stock solution), 0.60 mM benzoin (0.01 M stock of benzoin was made in 99.9% DMSO) and 0.35 mM NADH. The reaction was initiated by the addition of 0.5 μg of BAL and was recorded for 5 min at 340 nm and 30 °C.

Rapid-Scan Stopped-Flow Photodiode Array Experiments. (PDA) studies were carried out on an SX.18MV stopped-flow spectrophotometer from Applied Photophysics, Leatherhead, United Kingdom. A slit-width of 2 mm and a path length of 2 mm were used. Experiments were performed by mixing an equal volume of substrate and enzyme of intended concentrations. Typically, 1600 spectra were collected for each experiment with varied reaction times and the lowest time interval of 2.5 ms (see specific conditions for each experiment in the figure legends). PDA spectral data were deconvoluted using Pro-Kineticist Global kinetic analysis software version 4.21 from Applied Photophysics (Pro/K). In each deconvolution, singular value decomposition (SVD) of data sets was carried out using Pro/K, which yielded unbiased information about the minimum complexity of the chemical system, independent of model and indicating the number of independent components present. The SVDs generated in this manner were treated using the analysis tool of the same software to fit to a minimally complex model. The convergence was tested with the maximum number of iterations allowed by the software. The model validity was cross-checked using parameter minimization. The obtained fit spectra were cross-checked by analyzing the raw data sets using PeakFit version 9.0 from Systat. Sigma plot 2001 version 7.101 was used for the preparation of all the difference spectra and data analysis. Stopped-flow circular dichroism experiments were carried out on a π*-180 CDF Spectrometer from Applied Photophysics using a slit width of 2 mm and a path length of 10 mm at 30 °C. In a typical experiment, the required amount of BAL in buffer A [50 mM Tris-HCl (pH 8.0) containing 1 mM ThDP, 2.5 mM $MgSO_4$ and 10% v/v DMSO] was mixed with an equal volume of substrate/analogue solution at the desired concentration in the same buffer. The reactions were monitored for varied times (0.01–200 s), and data points were collected at varied time intervals (2.5–10 ms). The data were analyzed using Pro-K Global Analysis software. SVDs were determined where necessary. Data analysis used SigmaPlot 2001 version 7.101.

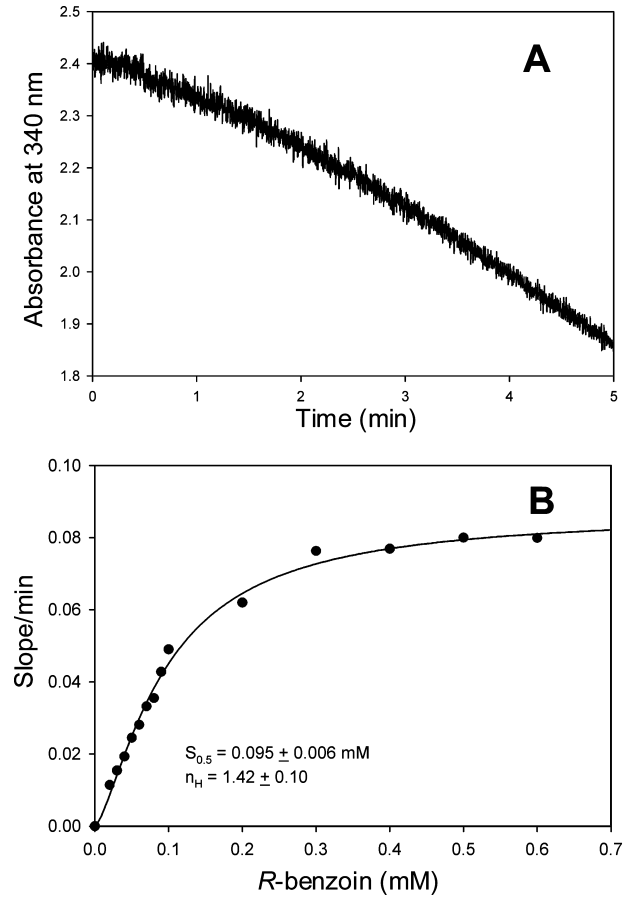


FIGURE 2: (A) Typical progress curve of the BAL reaction in the standard assay buffer showing lag phase. (B) Dependence of the reaction rate of benzaldehyde formation from (*R*)-benzoin by BAL at pH 8.0. Data were fitted according to the Hill equation (*37*). The BAL activity was measured as described under Experimental Procedures.

Circular dichroism titration experiments were carried out in a Chirascan spectrometer from Applied Photophysics. Spectra were recorded in the wavelength range of 290–500 nm using a 1 cm path quartz cell at 30 °C unless otherwise specified (see specific conditions for each experiment in the figure legends).

RESULTS

Studies of Benzaldehyde Lyase with Benzaldehyde and Benzoin. Assay of BAL Activity. This assay varies from those in the literature (*20, 22*) in that the benzoin stock solution is prepared in DMSO to keep the assay solution homogeneous. This created a reproducible continuous assay by coupling benzaldehyde production to its reduction by horse liver alcohol dehydrogenase/NADH. As seen in Figure 2A, there is a significant lag phase in the progress curve for benzaldehyde formation preceding steady state. The v_0 − [S] plot gave an $S_{0.5}$ = 0.095 mM and Hill coefficient n_H of 1.42, suggesting modest cooperativity (Figure 2B). Specific activities obtained using the standard BAL assay were between 30 and 40 μmol/min/mg, consistent with those obtained in earlier studies (*20, 22*).

Stopped-Flow Photodiode Array Studies between BAL and Benzoin Reveal Enamine formation. In a stopped-flow PDA experiment mixing BAL with (*R*)-benzoin, we observed the

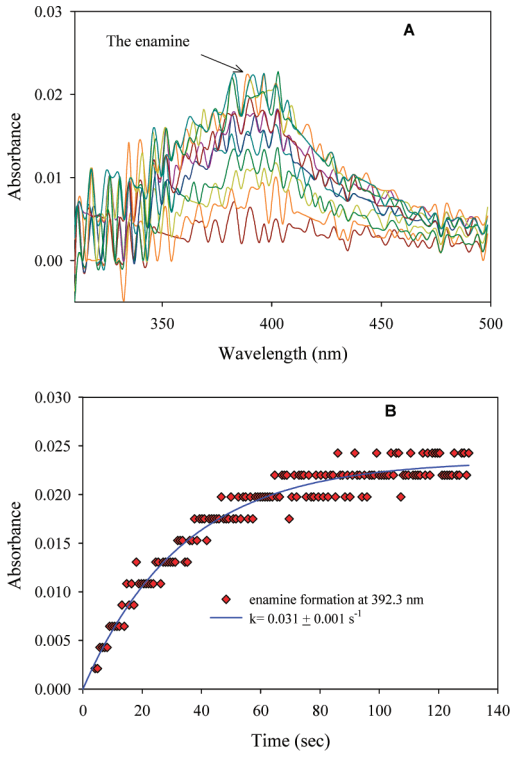


FIGURE 3: Stopped-flow PDA experiment on reacting (*R*)-benzoin with BAL. (A) Difference spectra showing enamine formation on mixing BAL (33.94 μM active-site concentration) in buffer A (see Experimental Procedures) with an equal volume of 4 mM (*R*)-benzoin in the same buffer at 20 °C. The reaction was monitored in the wavelength range indicated for 2 min, and spectra were recorded every 2.5 ms. (B) Rate of formation of enamine on BAL from (*R*)-benzoin from data in A. Data were fitted to a single exponential.

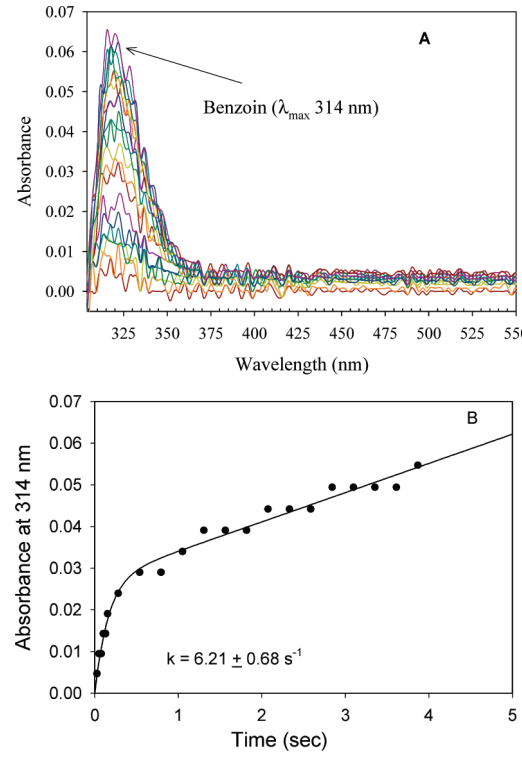


FIGURE 4: Stopped-flow PDA spectra resulting from the reaction of benzaldehyde with BAL. (A) BAL (33.94 μM active-site concentration) in buffer A was mixed with an equal volume of 20 mM benzaldehyde in the same buffer at 30 °C. The reaction was monitored in the indicated wavelength range for 16 s, and spectra were recorded every 2.5 ms. (B) Progress curve for benzoin formation from benzaldehyde on BAL from the reaction in A. Data were fitted to the equation: [Benzoin] = $v_s t + [\{(v_o - v_s)/k\} \cdot (1 - e^{-kt})]$, where v_o is the initial velocity, v_s is the steady-state velocity, and k is the rate constant ($v_o = 0.175 \pm 0.015$ A_{314}/s; $v_s = 0.007 \pm 0.0005$ A_{314}/s and $k = 6.21 \pm 0.68$ s^{-1}).

slow formation of a small absorbance with λ_{max} at 393 nm (Figure 3A), and the signal was stable for 2 min with a rate of intermediate formation of 0.031 s^{-1} (Figure 3B). This signal can be assigned to the enamine intermediate supported by both the model studies (*7, 8*) and the results with benzoylfomate and BAL below. The lag phase seen in the progress curve for benzaldehyde formation (Figure 2A) may be attributed to the slow rate of enamine formation.

Stopped-Flow Photodiode Array Studies between BAL and benzaldehyde Reveal No Intermediates. When BAL was reacted with benzaldehyde, no intermediate could be detected even at a 2.5 ms time resolution; only (*R*)-benzoin formation could be observed at 314 nm (Figure 4A). The rate of (*R*)-benzoin formation showed biphasic kinetics with an initial burst being followed by steady state product release (Figure 4B). The rate constant for formation of benzoin was 6.21 s^{-1}.

$^1H/^2H$ Kinetic Isotope Effects on the Reaction of Benzaldehyde with BAL. According to Scheme 1, the pathway for the conversion of benzaldehyde to benzoin involves the initial formation of HBThDP, followed by the dissociation of a proton at the C2α position forming the enamine, finally carboligation with a second benzaldehyde molecule forming DDEThDP, then benzoin release. With the exception of the enamine derived from benzoin, which is expected to have a λ_{max} near 380 nm (*7, 8*), the λ_{max} for intermediates such as DDEThDP and HBThDP should be lower than 260 nm, not enabling direct detection by electronic spectroscopy. We wished to test whether formation of the enamine is rate

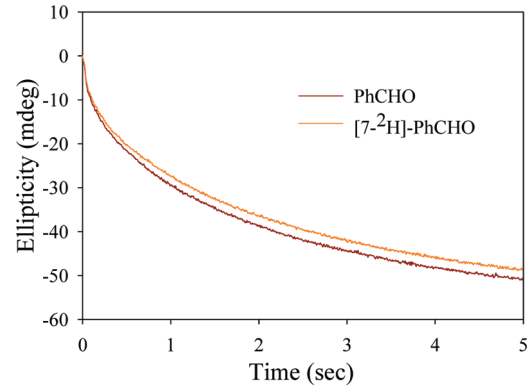


FIGURE 5: Determination of hydrogen kinetic isotope effect on the rate of formation of (*R*)-benzoin in the stopped-flow CD. BAL (0.08 mg/mL, 1.36 μM active site concentration) in buffer A was mixed with an equal volume of 20 mM benzaldehyde (or [7-^{2}H]-benzaldehyde) in the same buffer. The reaction was monitored at 313 nm in a 10 mm cell with 2 nm slit width for 5 s. Data points were collected every 2.5 ms. Data were fitted to $y = y_0 + a^*(1 - e^{-b^*x}) + c^*(1 - e^{-d^*x})$.

limiting, given the expected high pK_a found in models at Rutgers. We carried out the reaction shown in Figure 5 monitoring benzoin formation on a stopped-flow CD instrument. When BAL was reacted with [7-^{1}H]-benzaldehyde or [7-^{2}H]-benzaldehyde, the rate of benzoin production did not show a significant kinetic isotope effect (KIE). The rate constants for benzoin production with [7-^{1}H]-benzaldehyde

were 14.4 ± 0.3 s^{-1} and 0.7 ± 0.01 s^{-1}, whereas with [7-^{2}H]-benzaldehyde they were 12.6 ± 0.2 s^{-1} and 0.68 ± 0.01 s^{-1} (Figure 5). The k_H/k_D was 1.143 ± 0.03 for the faster phase and 1.0294 ± 0.0211 for the slower phase.

Studies of Benzaldehyde Lyase with Benzoylformic Acid and Phenylpyruvic Acid. Circular Dichroism Studies of BAL with Benzoylformate and Phenylpyruvate Reveal Formation of the Substrate−ThDP Covalent Intermediate via the 1′, 4′-Iminopyrimidine Tautomeric Form. A CD titration of BAL with 0.10– 9.5 mM benzoylformate (BF) at 4 °C (Figure 6A) led to the development of a positive CD band at 310 nm, which reached a limiting value ($S_{0.5\ \text{benzoylformate}}$ = 1.8 mM), indicating the formation of a covalent adduct between ThDP and BF, C2α-mandelylThDP. According to the CD spectra, this C2α-mandelylThDP adduct exists in its IP form (Figure 6A). Once the temperature of the reaction mixture was raised to 20 °C, time-dependent formation of (*R*)-benzoin was detected and was confirmed with the CD spectrum of authentic (*R*)-benzoin (Figure 6B). The compound (*R*)-benzoin gives rise to a negative CD band with maximum at 314 nm, similar to that recorded for the reaction mixture after BAL was removed (Figure 6C). The above data clearly indicate that BAL indeed catalyzes the decarboxylation of BF forming benzaldehyde and (*R*)-benzoin, the latter could be detected by CD. Earlier, formation of a low level of benzaldehyde was detected by HPLC when benzoylformate was added to BAL, but not when the BAL activity was measured using the coupled assay (*20*). The formation of 1′,4′-imino-MThDP displays saturation with increasing benzoylformate concentration, indicating that the complex was fully formed (Figure 6A, inset).

To avoid the formation of (*R*)-benzoin observed with benzoylformate, a similar experiment was carried out with phenylpyruvate (Figure 7). The data indicated that phenylpyruvate forms the covalent adduct with ThDP on BAL as well. On titration of BAL with phenylpyruvate, a positive band at 307 nm developed and reached a limiting value, ($S_{0.5\ \text{phenylpyruvate}}$ = 0.53 mM) indicating that the adduct with ThDP exists in the IP tautomeric form. A parallel control of the BAL activity revealed about 41% of activity for the BAL treated with 3 mM phenylpyruvate (the maximum concentration of phenylpyruvate used for CD titration), indicating that at least half of the active centers of BAL were being saturated with phenylpyruvate.

Stopped-Flow Photodiode Array Studies of BAL with Benzoylformate Also Reveal Formation of the Enamine Intermediate. Further proof that BAL could function as a decarboxylase was obtained by reacting BF with BAL in the stopped-flow PDA instrument, revealing very slow formation of the intermediate at 392 nm (Figure 8A), assigned on the basis of model studies (*7, 8*), to the enamine (Scheme 1). The rate of formation of the enamine showed an initial lag phase followed by an exponential rise to maximum (Figure 8B) with a rate constant of 0.008 s^{-1}. The experiment provides strong support for the decarboxylation of BF by BAL.

Studies of Benzaldehyde Lyase with (E)-2-Oxo-4(pyridin-3-yl)-3-butenoic Acid and (E)-3-(Pyridine-3-yl)acrylaldehyde. Stopped-Flow Photodiode Array Studies of BAL with 3-PKB Reveal the Formation of C2α-mandelylThDP Analogue. To investigate whether 3-PKB is a substrate for BAL, a stopped-flow experiment was carried out by mixing BAL (33.2 μM active-site concentration) with an equal volume of 10 mM 3-PKB at 30 °C. The reaction was monitored in the wavelength range of 300–600 nm for varied time periods (4–256 s), and the spectra were recorded every 2.5 ms (Figure 9A). The difference spectra in Figure 9 (obtained by using the first spectrum as reference and by subtracting it from

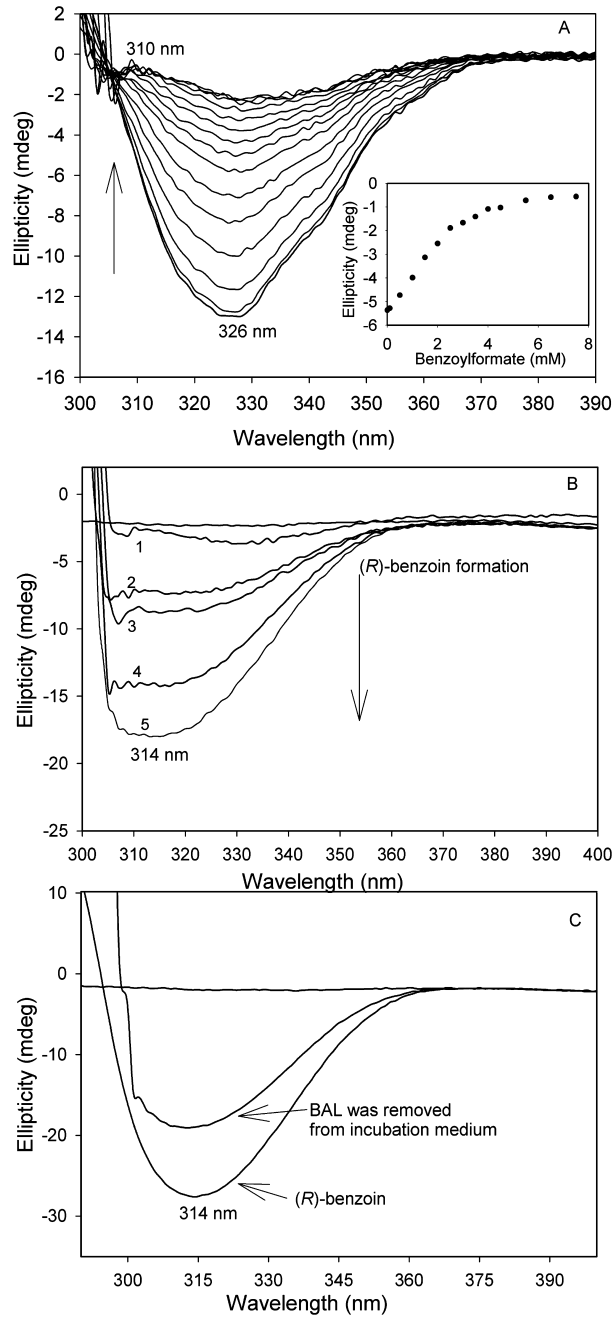


FIGURE 6: CD titration of BAL by benzoylformate. (A) To BAL (2.5 mg/mL, concentration of active centers = 42.4 μM) was added benzoylformate (0.10–9.5 mM) in 50 mM Tris-HCl (pH 8.0) containing ThDP (0.150 mM) and $MgSO_4$ (1.0 mM) at 4 °C. The arrow indicates the direction of change at 310 nm with increasing concentrations of benzoylformate. Inset: Changes in ellipticity at 310 nm on addition of benzoylformate. (B) CD detection of the formation of (*R*)-benzoin from benzoylformate by BAL at 20 °C. Spectrum 1, the reaction mixture containing BAL and benzoylformate (9.5 mM) recorded at 4 °C. Spectra 2–5, the same mixture as in spectrum 1 but recorded at 20 °C within 35 min (2), 60 min (3), 120 min (4), and 154 min (5) of incubation. The arrow indicates the time-dependent formation of (*R*)-benzoin. (C) CD spectra of (*R*)-benzoin and the reaction mixture after BAL was removed.

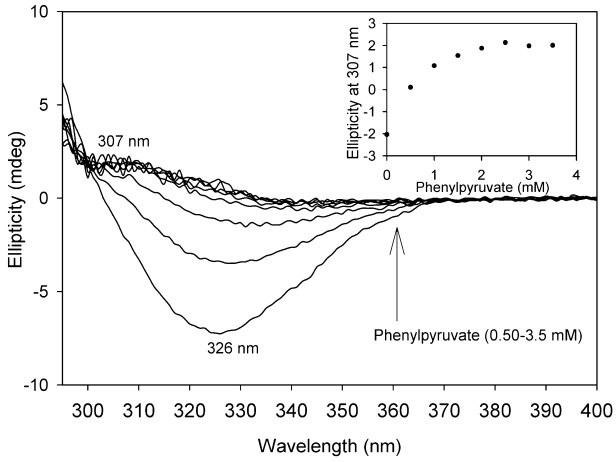


FIGURE 7: CD titration of BAL by phenylpyruvate. To BAL (1.5 mg/mL, concentration of active centers = 25.4 μM) in 50 mM Tris-HCl (pH 8.0) containing ThDP (150 μM) and $MgSO_4$ (1 mM) was added phenylpyruvate (0.5–3.5 mM) at 30 °C. The inset shows the dependence of the ellipticity at 307 nm (due to the 1′,4′-iminopyrimidine tautomer of the adduct between phenylpyruvate and ThDP) on the concentration of phenylpyruvate.

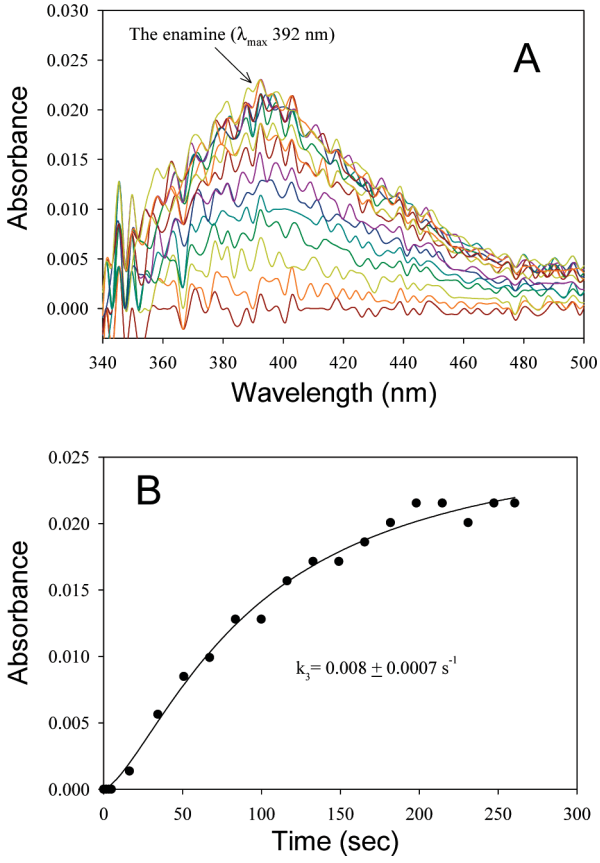


FIGURE 8: Difference spectra showing enamine formation from benzoylformate on BAL. (A) BAL (33.94 μM active-site concentration) in buffer A was mixed with an equal volume of 20 mM benzoylformate in the same buffer at 30 °C. The reaction was monitored in the wavelength range indicated for 5 min, and spectra were recorded every 25 ms. (B) Rate of formation of enamine from benzoylformate on BAL; conditions as described in A. The rate of formation showed an initial lag phase followed by an exponential rise to maximum with a rate constant of 0.008 s^{-1}.

every subsequent spectrum) displayed a broad absorbance band with two humps at λ_{max} of 477 nm and λ_{max} of 437 nm. The development of these absorbance bands was very slow, and the bands were stable even after 20 min. Comparing with similar bands in YPDC and BFDC, the absorbance at λ_{max} of 477 nm could be assigned as a MThDP analogue (MThDP*, the predecarboxylation analogue formed between ThDP and 3-PKB), whereas the second absorbance at λ_{max} of 437 nm needed to be assigned. That the two maxima probably referred to the same electronic transition is supported by the finding that their rate of formation was similar (0.081 ± 0.0004 s^{-1}; 0.0041 ± 0.0003 s^{-1} and 0.081 ± 0.0006 s^{-1}; 0.0039 ± 0.0001 s^{-1}) and that they appeared simultaneously (Figure 9B).

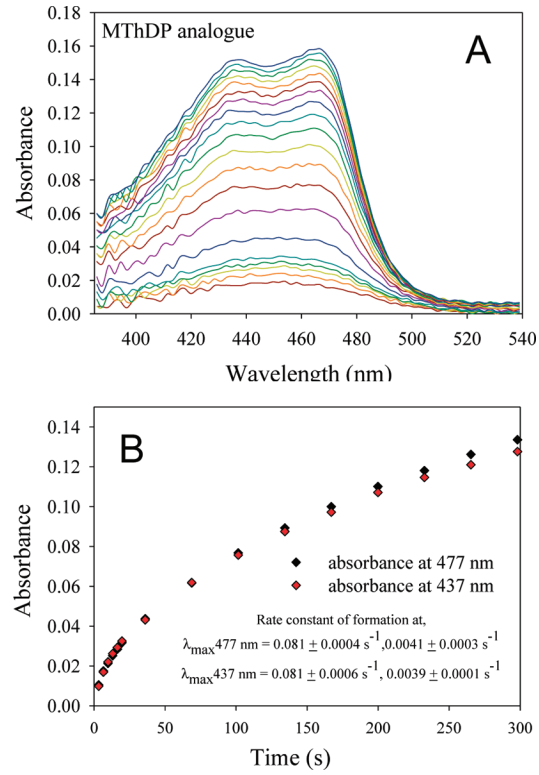


FIGURE 9: Difference PDA spectra resulting from mixing BAL and 3-PKB showing MThDP* formation. (A) BAL 2 mg/mL (33.2 μM active-site concentration) in buffer A was mixed with an equal volume of 20 mM 3-PKB in the same buffer at 30 °C. The reaction was monitored in the wavelength range shown for 10 min, and spectra were recorded every 5 ms. (B) Rate constants for the formation of the two absorbance bands observed at 477 and 437 nm in the PDA experiments in A. The data at the specific λ were treated in SigmaPlot and fitted to the following equation: $y = y_0 + a^*(1 - e^{-b^*x}) + c^*(1 - e^{-d^*x})$.

Circular Dichroism Titration of BAL with 3-PKB Confirms the Formation of the MThDP.* To obtain further insight into the reaction of 3-PKB with BAL, a CD titration experiment was carried out. Upon titration of BAL with 3-PKB, a negative CD band was formed at λ_{max} 477 nm, whose amplitude displays saturation at 6 mM 3-PKB; at the same time, a broad positive band appeared with λ_{max} of 437 nm, also displaying saturation at 6 mM 3-PKB (Figure 10A and B). Both the PDA and CD experiments confirm that 3-PKB can form a covalent ThDP-bound predecarboxylation intermediate but that this MThDP* is stable and that decarboxylation does not take place.

Stopped-Flow Photodiode Array Studies of BAL and PAA Reveal the Formation of the Aldehyde−ThDP Covalent Intermediate. It is well established that BAL can accept a broad range of aromatic aldehydes as substrates (*2*), and we next tested the decarboxylated product of 3-PKB, (*E*)-3-(pyridine-3-yl)acrylaldehyde (PAA), as the substrate from

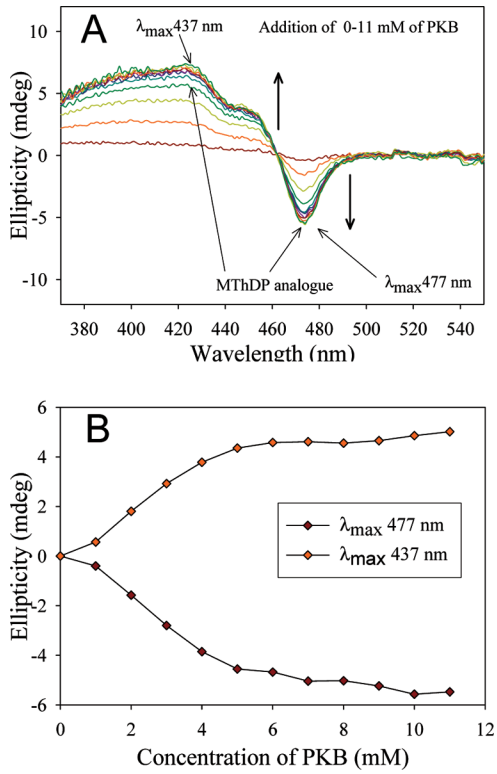


FIGURE 10: CD spectra resulting from the titration of BAL with 3-PKB. (A) BAL (2 mg/mL) (33.2 μM active-site concentration) was titrated with 0–11 mM 3-PKB in buffer A at 30 °C in a Chirascan CD instrument using a 1 cm path-length quartz cell. The arrows indicate the direction of change with increasing concentration of 3-PKB. (B) Dependence of the amplitude of the bands at 477 and 437 nm on the concentration of 3-PKB in A.

the aldehyde direction. The stopped-flow PDA spectra on mixing BAL with PAA display a broad absorbance formed with a λ_{max} of 456 nm (Figure 11A). The shape of the band and the high extinction coefficient are in good agreement with that expected of a charge transfer band. The results are similar to those observed when mixing YPDC or BFDC with PAA, enabling us to assign the band to an adduct formed between PAA and ThDP, analogous to HBThDP. The rate of formation of the HBThDP analogue was approximately 30 s^{-1} (Figure 11B), the signal reached maximum within 0.2 s, and the signal was stable for more than 15 min.

CD Studies of the Reaction between BAL and PAA Confirm the Formation of the Aldehyde−ThDP Adduct. When BAL (2.5 mg/mL) was titrated with 0–1 mM PAA, the resulting spectra were similar to those observed in the CD titration experiments of BAL and 3-PKB. Formation of a negative CD band at 477 nm was accompanied by the formation of a positive band at 434 nm (Figure 11C). Both bands were assigned to the HBThDP analogue.

Both the PDA and CD experiments confirm that PAA can form a covalent ThDP-bound intermediate, an analogue of HBThDP, and that the analogue is stable. Apparently, the C2αH is not dissociated to form the enamine from PAA. The rate of formation of the PAA−ThDP adduct is very much faster than that of the 3-PKB−ThDP adduct, emphasizing that the active center of BAL has not evolved to bind negatively charged substrates or analogues.

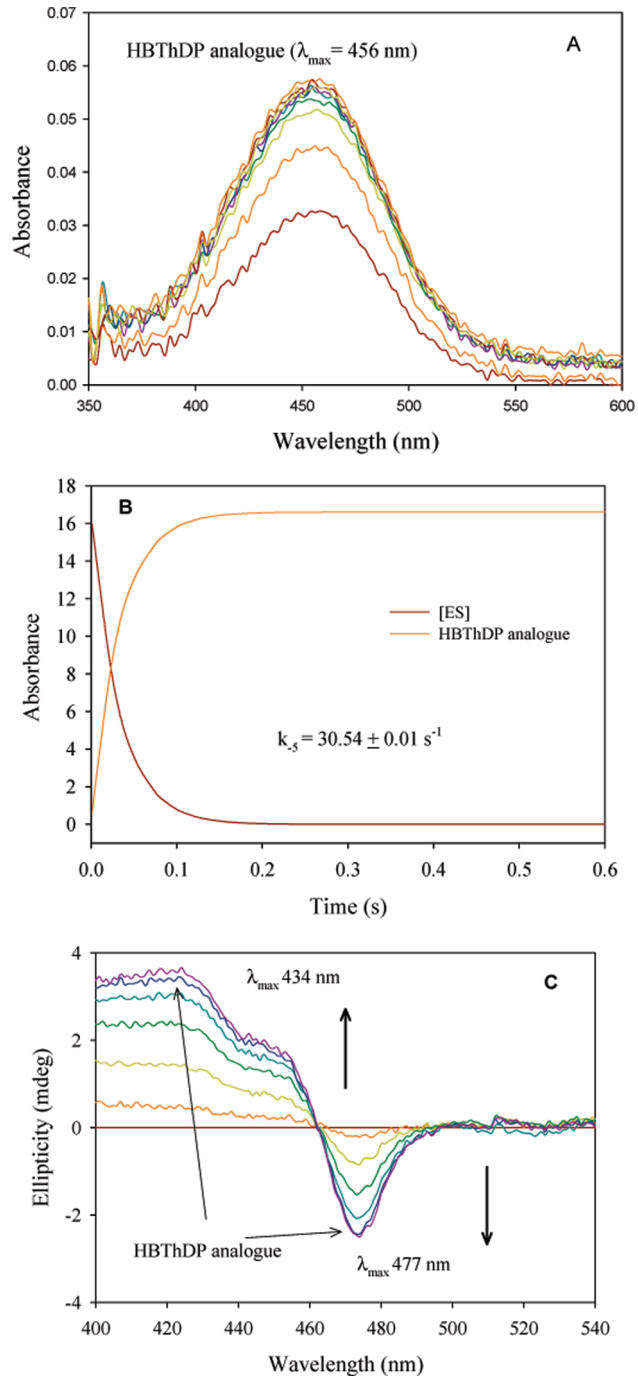


FIGURE 11: Reaction of PAA with BAL. (A) Difference spectra of 2 mg/mL BAL (33.2 μM active-site concentration) in buffer A mixed with an equal volume of 20 mM PAA in the same buffer at 30 °C. The reaction was monitored for 10 min, and the spectra were recorded every 2.5 ms. (B) Rate of formation of the HBThDP analogue at 456 nm for the reaction in A. ES on the figure refers to BAL saturated with PAA. PDA spectral data were deconvoluted as described in Experimental Procedures. Data were fitted to a single exponential. The rate constant k_{-5} refers to Scheme 1. (C) CD spectra resulting from mixing BAL with PAA. BAL (2 mg/mL, 33.2 μM active-site concentration) was titrated with 0–1 mM PAA in buffer A at 30 °C in a Chirascan CD instrument using a 1 cm path-length quartz cell in the indicated wavelength range.

DISCUSSION

The group at Rutgers has had a long-term interest in the detection of ThDP-bound and ThDP-related intermediates in the catalytic cycle of yeast pyruvate decarboxylase (YPDC) (*4, 6, 14, 16*), the E1 component of the pyruvate

dehydrogenase complex from *E. coli* (E1ec *5, 6, 15, 16*) and from human sources (E1h *18, 19*), BFDC (*19, 23, 24*), pyruvate oxidase (POX *18, 19*) and BAL (*19*). Using CD spectroscopy in conjunction with sodium methyl acetylphosphonate (NaMAP), an analogue of pyruvate, or phosphonolactylthiamin diphosphate (PLThDP), a stable analogue of C2α-lactylThDP resulting from adding ThDP to MAP, spectroscopic evidence was obtained for the existence of the 1′,4′-iminoThDP tautomer (IP) on E1ec, E1h, YPDC, POX and some of their variants (*15, 16, 18, 19, 25*). For example, with E1h two CD bands were detected on addition of ThDP by itself, even in the absence of substrate analogue (*18*). On the basis of both model studies and six ThDP-dependent enzymes studied at Rutgers, these two bands were assigned to the different tautomeric forms of ThDP: (a) the negative band at 320–330 nm to a charge-transfer band resulting from the interaction of the 4′-aminopyrimidine moiety and the thiazolium ring of ThDP, providing a signature for the AP form of ThDP bound to the enzyme; (b) the positive band at 300 –305 nm was assigned to the IP form of ThDP (*14, 17*).

Interestingly, the CD spectra of E1ec, E1h, YPDC, POX, BFDC and BAL present some differences, suggesting the possible contribution of different active center residues to the observed electronic spectral signatures. In this respect, BAL is of special interest since its active center is formed by nonpolar aliphatic and aromatic residues (Figure 1), with only a few charged amino acid residues being present (*3, 20*).

As was recently shown (*19*), the CD spectrum of BAL at pH 8.0 revealed the presence of the AP form of ThDP (the negative CD band at 326 nm), while the IP form of ThDP could not be detected under these conditions. The additional presence of the APH^+ form could not be excluded, as we do not have a CD signature for this form at present. On reducing the pH from 8.0 to 7.0, the negative CD band at 326 nm was no longer observed on BAL, suggesting the transition from the AP to APH^+ form, and a pK_a of 7.42 was calculated for the $APH^+$to (AP+IP) transition. The pH-dependent changes of the CD spectra correlate well with the pH-dependence of BAL activity, indicating that amino acid residue (s) from the active center contribute to the APH^+ to (AP+IP) transition, most likely the residue E50. In this study we wished to directly observe ThDP-bound intermediates on BAL.

BAL-Catalyzed Formation of Benzoin from Benzaldehyde and the Rate-Limiting Step in the Ligase Reaction. In the stopped-flow PDA studies of the reaction between BAL and benzaldehyde, no intermediates could be detected even on the instrument time scale of 2.5 ms, but benzoin formation could be monitored at $\lambda_{max} = 314$ nm.

Following these observations, we decided to determine the kinetic isotopic effect (KIE) on benzoin production starting with benzaldehyde, using [7-^{2}H]benzaldehyde along with unlabeled material. The rate constants for formation of benzoin starting from benzaldehyde and [7-^{2}H]benzaldehyde were very similar with a $k_H/k_D = 1.14$. While the [7-^{2}H]benzaldehyde reacts more slowly, clearly the magnitude of the KIE is small but normal (>1.0), i.e., it is outside the experimental error. According to the presumed mechanism in Scheme 1, with this experiment, we should be able to assign the rate-limiting step as follows: (1) addition of the ThDP ylide to the aldehyde carbon (converts an sp^2 hybridized carbon to an sp^3 hybridized one) should be accompanied by a small inverse secondary α-^{2}H KIE; (2) deprotonation of HBThDP derived from [7-^{2}H]benzaldehyde to the enamine is the step most sensitive to isotopic substitution and should carry a large primary KIE of magnitude 4–6 according to model studies (*7, 8*); (3) finally, addition of the enamine to the second benzaldehyde may again show a small inverse secondary α-^{2}H KIE. Therefore, given the observed value, we conclude that the release of benzoin is likely to be the slowest step in the reaction.

Observation of ThDP-Bound Intermediates on Benzaldehyde Lyase. In this study, for the first time, we identified and determined the kinetic fate of three ThDP-bound covalent intermediates on BAL (HBThDP, enamine and MThDP) using stopped-flow and CD titration experiments.

Observation of the Enamine Intermediate. Interestingly, we could generate the enamine intermediate on BAL with two different approaches. (1) In the stopped flow PDA experiments, a small absorption with λ_{max} of 393 nm is formed, which is derived from the real substrate, benzoin. (2) Alternatively, we could take advantage of the common observation that carboligases using ThDP also do give decarboxylase activity (see use of β-hydroxypyruvate as a source of the enamine on TK in ref *26*). Starting with benzoylformate, we observed the formation of an absorbance with λ_{max} of 393 nm on BAL in the stopped flow PDA experiments. Formation of the band showed a lag phase followed by an exponential rise to maximum. The rate constant for the formation of the enamine from benzoylformate on BAL was very slow (0.008 s^{-1}), and it was stable for more than 4 min. The slow rate of enamine formation could be attributed to the weak decarboxylase activity of BAL. In other experiments, the enamine was also observed with the same λ_{max} of 393 nm when BFDC was reacted with the product of its reaction benzaldehyde (*11*). Chemical model studies (*7, 8*) clearly confirm that this absorbance could only pertain to the enamine.

We wish to emphasize the importance of the direct observation of the enamine to ThDP enzymes. We have shown over a 25 year period with several enzymes [YPDC (*29–31*), BAL (this study) and BFDC (*11, 23, 24*)] using a variety of conjugated 2-oxo acids (such as *m*- and *p*-X-$C_6H_4CH{=}CHCOCOOH$ with a variety of X groups on YPDC, *p*-NO_2-$C_6H_4COCOOH$ on BFDC and YPDC, and now with 3-PKB on YPDC, BAL and BFDC) that the enamine absorbance appears with a λ_{max} where predicted by chemical models in which the enamine is generated in an unambiguous manner (*7, 8, 30*). Equally importantly, in several cases it could be established that on the enzyme the enamine is formed at rates consistent with the turnover number for the true substrates, i.e., through decarboxylation, these substituted pyruvate analogues had no impact on the kinetic barriers (see for example YPDC in ref *31*). These results are most consistent with a planar enamine structure, at least when there is extensive conjugation such as in benzoylformate or other analogues in this group (Scheme 1).

X-ray structural evidence on this point has been less clear: a paper on transketolase (*26*) and pyruvate oxidase (*32*) suggested the presence of a planar enamine, while papers on oxalyl-CoA decarboxylase (*33*) and the E1 component of the human branched chain 2-oxo acid dehydrogenase complex (*34*) suggested a pyramidal distortion at the C2α

atom, more consistent with the contribution of the carbanionic (zwitterionic) resonance structure to the resonance hybrid. Because of the difficulty in observing the enamine derived from pyruvate and other aliphatic 2-oxo acids (expected λ_{max} between 290 and 295 nm according to models at Rutgers *5, 6*), direct confirmation of this notion by electronic spectroscopic methods is more difficult. Hence, whether the C2α atom of the enamine derived from aliphatic 2-oxo acids is planar or pyramidal is not settled. That the C2−C2α bond suffers distortion with respect to the thiazolium plane in predecarboxylation intermediates and other C2α-tetrasubstituted ThDP-bound intermediates has now been suggested for several enzymes with some confidence [the E1 component of the *E. coli* pyruvate dehydrogenase complex (*35*), pyruvate oxidase (*32*), and on transketolase (*36*)], but this may or may not have an impact on the structure of the postdecarboxylation intermediate.

Observation of the 1′,4′-Imino-MThDP Intermediate in BAL with Benzoylformate and Phenylpyruvate. In CD titration experiments, the formation of a positive band at λ_{max} of 310–312 nm was observed on incremental addition of benzoylformate to BAL at 4 °C. The reaction was carried out at a lower temperature to further slow the already miniscule decarboxylase activity of BAL. In addition to the 1′,4′-iminoMThDP, we also observed the formation of a negative band at λ_{max} of 329 nm indicating the formation of a Michaelis complex (since the negative band never disappeared but rather was converted to a different one with lower molar ellipticity). Such a negative CD band in this wavelength region representing a noncovalent Michaelis complex had been observed earlier for the interaction of MAP with YPDC (*16*), and more recently with active center loop variants of the E1ec even with pyruvate (*27*). The observations of the MThDP with benzoylformate were confirmed via parallel experiments with phenylpyruvic acid, which also gave the positive CD band at 312 nm, characteristic of the 1′,4′-iminoThDP with the C2(2-carboxy-2-hydroxy)phenethylThDP substituent. We believe that this is the first instance of the observation of the MThDP intermediate on an enzyme.

Detection of MThDP and HBThDP Analogues in the Reaction between BAL and 3-PKB or PAA. In the stopped-flow PDA studies of the reaction between BAL and 3-PKB, a stable broad absorbance with two humps at $\lambda_{max} = 477$ nm and $\lambda_{max} = 437$ nm were observed. Earlier, we used this substrate with YPDC and BFDC (*11*), where we had a different experience: only a single absorbance with $\lambda_{max} = 473$ nm was first observed, which was transformed with time into a band of $\lambda_{max} = 433$ nm. On BAL, the absorbances at $\lambda_{max} = 433$ nm and at $\lambda_{max} = 473$ nm were formed with the same rate constant; hence, they must represent the same species. As the intermediate was stable, we could carry out a steady-state experiment in the CD, where the bands also appeared simultaneously, and the formation of the bands showed similar dependence on the concentration of 3-PKB. Interestingly, the VIS absorbance at $\lambda_{max} = 477$ nm corresponds to a negative CD band, whereas the VIS band at $\lambda_{max} = 437$ nm corresponds to a positive CD band. From all the evidence, we conclude that both bands belong to the same MThDP-type intermediate. The stability of the band could be attributed to the slow decarboxylase activity of BAL. The formation of the band at $\lambda_{max} = 437$ nm could be attributed to an additional charge transfer band with an aromatic amino acid residue in the highly hydrophobic active center of BAL.

In the stopped-flow PDA studies of the reaction between BAL and PAA, a stable broad absorbance at $\lambda_{max} = 456$ nm was observed. Earlier, we used this substrate with BFDC (*23, 24*), where we observed formation of the HBThDP analogue at $\lambda_{max} = 473$ nm, the same wavelength where the MThDP* derived from 3-PKB absorbs. However, in the CD experiment we observed two different bands, as in the reaction between BAL and 3-PKB, a negative one at $\lambda_{max} = 477$ nm and a positive one at $\lambda_{max} = 437$ nm. On the basis of the evidence, we assign the absorbance band $\lambda_{max} = 456$ nm to the HBThDP-like intermediate, which arises because of the overlap of the bands at $\lambda_{max} = 477$ nm and $\lambda_{max} = 437$ nm. The rate constant of the formation of the HBThDP-like intermediate was 30 s^{-1}, which is consistent with our observation that aldehydes are better substrates for BAL than α-hydroxyketones.

We note that, while ThDP-bound covalent intermediates are being formed with both 3-PKB and PAA, the reactions do not proceed further; therefore, these compounds only inform about the formation of the tetrahedral ThDP-bound intermediates, not about the enamine.

SUMMARY

Further evidence was obtained for the presence of the 1′,4′-iminopyrimidine tautomeric form of the ThDP-bound intermediates when there is a C2α atom with four substituents. With only three substituents at the C2α atom (such as the enamine), presumably the APH^+ form predominates for which we have no spectroscopic signature. For the first time, the enamine intermediate could be detected by electronic spectroscopy on the enzyme from a true substrate, and the results gave clear indications that it is planar and highly conjugated. Importantly, we also established experimental conditions under which all three covalent intermediates are relatively stable on BAL and could potentially be crystallized. Finally, we could determine the rate-limiting step of the reaction in the benzoin formation direction. The research on BAL further underlines the fact that all thiamin-bound intermediates, as well as different tautomeric and ionization states of ThDP, are chiral when enzyme bound. We further suggest, that CD experiments should serve as very important confirmatory and complementary evidence for the ThDP-bound intermediates seen, or proposed on the basis of X-ray structural evidence, especially if and until hydrogen positions can be defined with precision.

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