Single-Turnover Studies on Brewer's Yeast Pyruvate Decarboxylase: C(2)-Proton Transfer from Thiamin Diphosphate[1]

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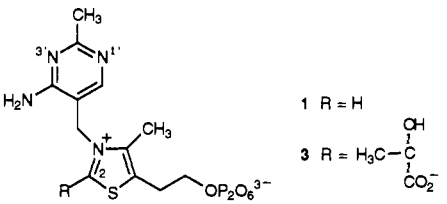
Received April 20, 1993®

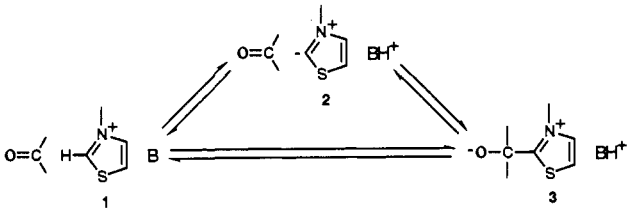
Abstract: Rate constants for formation of acetaldehyde from pyruvate catalyzed by the thiamin diphosphate (TDP)-dependent enzyme pyruvate decarboxylase (PDC; EC 4.1.1.1) from *Saccharomyces carlsbergensis* were determined under single-turnover conditions at 30 °C in 100 mM sodium 2-(*N*-morpholino)ethanesulfonate buffer (pH 6.00) containing 100 mM pyruvamide, 10 mM $MgSO_4$, and 12.5 μM sodium pyruvate. Observed rate constants in the range k_{obsd} = 2.5–6.7 s^{-1} for 33–104 μM (8–15 mg mL^{-1}) pyruvamide-activated PDC agree with values of k_{obsd} calculated by numerical integration with microscopic rate constants derived previously from steady-state kinetic isotope effects. The observed rate constant k_{obsd} = 6.7 ± 0.4 s^{-1} is independent of the concentration of pyruvamide-activated PDC in the range 104–150 μM. The decrease in the concentration dependence of the observed rate constants at >104 μM PDC is consistent with either a change in rate-limiting step or complex formation involving the reactants. There is little or no primary kinetic isotope effect, $(k_H/k_D)_{obsd} \leq 1.2$, for C(2)-hydron exchange from PDC-bound TDP for 33–104 μM pyruvamide-activated PDC. This provides evidence against rate-limiting C(2)-proton transfer between C(2)–H in PDC-bound TDP and a catalytic base with $-7 \leq \Delta pK_a$ (= $pK_a^{BH} - pK_a^{C(2)H}$) ≤ 7 to form a discrete ylide intermediate during catalysis by PDC.

Transfer of the C(2) proton from the thiazolium ring of thiamin diphosphate (TDP) (**1**) forms a reactive thiazolium ylide (**2**, Scheme I), which is both a potent carbon nucleophile and a reasonably stable leaving group.[2] The ylide (**2**) derived from TDP has been implicated in aldol-type addition reactions between TDP and carbonyl compounds catalyzed by several TDP-dependent enzymes and is a putative intermediate during catalysis of acetaldehyde formation by the TDP-dependent enzyme pyruvate decarboxylase (PDC) (2-oxo-acid carboxy-lyase; EC 4.1.1.1).[2,3]

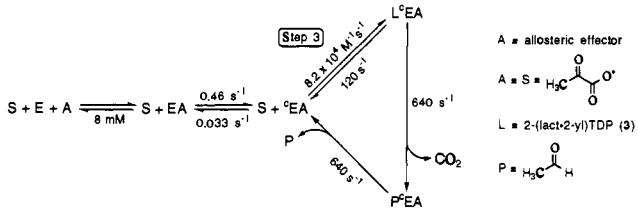


Primary ^{13}C and secondary kinetic isotope effects were used to establish the steady-state mechanism of brewer's yeast PDC shown in Scheme II.[3c,e] The addition of TDP to pyruvate to form 2-(lact-2-yl)TDP (**3**) (Scheme II; step 3) was shown to be 84 ± 4% rate limiting, while the decarboxylation and product-release steps are 16 ± 4% rate limiting.[3c] The relative magnitudes of the kinetic barriers within the addition step (step 3) are incompletely understood. It is not known whether enzyme-catalyzed proton abstraction from TDP to form the ylide is rate limiting for ylide addition to the carbonyl group in a stepwise enzymatic mechanism (Scheme I; upper path), whether TDP addition occurs in a one-step, concerted enzymatic mechanism (Scheme I; lower path) because of the relative instability of the carbanion,[4-6] or if a rate-limiting conformational change precedes addition of TDP to the substrate carbonyl group.

Scheme I



Scheme II



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® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

(1) Dedicated to the memory of Donald R. Cowell (1927–1993). This research was supported in parts by grants from the National Institutes of Health (GM 42878), the American Cancer Society (JFRA-213), and a Biomedical Research Support Grant to Johns Hopkins University (2S07-RR05445). Support was provided for E.J.C. by a National Institutes of Health Training Grant (5T32ES07141).

(2) Kluger, R. In *The Enzymes*, 3rd ed.; Sigman, D. S., Ed.; Academic Press: New York, 1992; Vol. 20, pp 271–315. See pp 307–312.

(3) (a) Alvarez, F. J. *Diss. Abstr. Int., B* **1986**, *46*, 3442–3443. (b) Alvarez, F. J.; Schowen, R. L. In *Thiamin Pyrophosphate Biochemistry*; Schellenberger, A., Schowen, R. L., Eds.; CRC Press: Boca Raton, FL, 1988; Vol. I, pp 101–112. (c) Alvarez, F. J.; Ermer, J.; Hübner, G.; Schellenberger, A.; Schowen, R. L. *J. Am. Chem. Soc.* **1991**, *113*, 8402–8409. (d) Huhta, D. W.; Heckenthaler, T.; Alvarez, F. J.; Ermer, J.; Hübner, G.; Schellenberger, A.; Schowen, R. L. *Acta Chem. Scand.* **1992**, *46*, 778–788. (e) We assume addition of TDP to the keto group of pyruvate is complete in the enzymatic state L°EA in Scheme II. The role of **3** in PDC catalysis has been considered in some detail (Kluger, R.; Smyth, T. *J. Am. Chem. Soc.* **1981**, *103*, 1214–1216).

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We have used single-turnover experiments on brewer's yeast PDC to determine whether addition of TDP to pyruvate involves rate-limiting ionization of the C(2) position of TDP. In this paper we show that rate constants for acetaldehyde formation catalyzed by PDC under single-turnover conditions agree with modeled time courses using the microscopic rate constants reported by Schellenberger and Schowen and their co-workers.[3c] The absence of a significant primary kinetic isotope effect for C(2)-hydron[7] abstraction from PDC-bound TDP provides (1) evidence against rate-limiting C(2)-proton transfer between TDP and a catalytic base with $-7 \leq \Delta pK_a$ ($= pK_a^{BH} - pK_a^{C(2)H}$) ≤ 7 to form a discrete ylide intermediate during catalysis by PDC and (2) evidence consistent with a concerted enzymatic mechanism, a stepwise enzymatic mechanism involving rapid equilibrium formation of the ylide as a discrete intermediate followed by rate-limiting C–C bond formation, or a rate-limiting conformational change. We suggest that the small intrinsic barrier for C(2)-hydron exchange from thiazolium ions, which gives a large change in transition-state structure[6] and in the observed primary kinetic isotope effect as the acidity of the carbon acid or catalytic base is changed, limits the range of measurable primary kinetic isotope effects to cases where $-7 \leq \Delta pK_a \leq 7$ on TDP-dependent enzymes.

Experimental Section

Materials. All chemicals were of analytical or reagent grade and were used without further purification unless otherwise stated. All water was prepared on a four-bowl Milli-Q water system including an Organex-Q cartridge (Millipore). Benzylidenemalanonitrile was recrystallized from 1-propanol: mp 83–85 °C. Pyruvamide was synthesized by acid hydrolysis of pyruvonitrile and purified by sublimation: mp 127 °C sub.[8] (2,4-Dinitrophenyl)hydrazones of butyraldehyde (mp = 108–111 °C (lit. 114 °C)), acetaldehyde (mp = 156–158 °C), pyruvate (mp = 219–221 °C (lit. 216 °C)), and pyruvamide (mp = 223–225 °C) were prepared as described previously[9] for use as HPLC standards. [Thiazole-2-D]TDP was prepared from [thiazole-2-H]TDP by exchange in D_2O (Aldrich, ≥99.9 atom % D), and ^1H NMR examination of the exchanged TDP indicated ≥98% C(2)–D in the product.[10] Stock solutions of [thiazole-2-D]TDP in D_2O contained 100 mM sodium 2-(*N*-morpholino)-ethanesulfonate (MES) buffer (pD 6.35), 10 mM $MgSO_4$, and 100 mM pyruvamide. Stock solutions of [thiazole-2-H]TDP in H_2O contained 100 mM sodium MES buffer (pH 6.00), 10 mM $MgSO_4$, and 100 mM pyruvamide. Brewer's yeast holo-PDC (45–60 unit mg^{-1})[11] and apo-PDC were prepared as described previously.[12] "Unresolved" PDC containing the α_4 and $\alpha_2\beta_2$ isozymes was used in the experiments reported here.[13]

Methods. Rapid-quench kinetic experiments with pyruvamide-activated PDC were performed with a KinTek Instruments Model RQF-3 rapid-quench-flow apparatus thermostated at 30 °C. "Reaction buffer" refers to 100 mM sodium MES buffer (pH 6.00) in H_2O containing 10 mM $MgSO_4$ and 100 mM pyruvamide unless otherwise stated. A typical experiment was initiated by the addition of 5 μL of 200 mM [thiazolium C(2)–L]TDP (L = H or D)[7] to 95 μL of apo-PDC in reaction buffer at room temperature ($t_1 = 0$); the final concentrations were 16–72 mg mL^{-1} PDC, 10 mM TDP, 100 mM pyruvamide, and 10 mM $MgSO_4$. The apo-PDC/cofactor solution was stirred with a pipette tip, taken up in a 100-μL gas-tight Hamilton syringe (No. 1710-TEFLL), quickly loaded into a sample loop, and incubated for a total reconstitution time of t_1 = 30 s to allow complete recombination of apo-PDC with [thiazolium C(2)–L]TDP and Mg^{2+}. The other sample loop contained 25 μM sodium pyruvate in reaction buffer. The drive syringes were filled with reaction buffer, and the quench syringe was filled with 1 M HCl. The decarboxylation reaction was initiated by mixing the contents of the sample loops to obtain a solution containing 12.5 μM sodium pyruvate and 8–36 mg mL^{-1} (33–150 μM) PDC that was allowed to react for a time t_2 (0.007 ≤ t_2 ≤ 5 s) before the reaction was acid-quenched. The 165–280 μL samples were collected in 1.5-mL microcentrifuge tubes, and 1 M HCl was added to bring all samples to a total volume of 300 μL. The quenched samples were centrifuged for ≥30 s at 16 000*g*, the supernates were transferred to 1.5-mL microcentrifuge tubes, 8 μL of 0.8 M (2,4-dinitrophenyl)hydrazine hydrochloride in Me_2SO and butyraldehyde (2,4-dinitrophenyl)hydrazone (internal standard) was added to the supernates, and the "spiked" solutions were stored in an ice–water bath before HPLC determination of the (2,4-dinitrophenyl)hydrazones of acetaldehyde and butyraldehyde.

Acetaldehyde produced at each time point (t_2) (≤3.75 nmol) was determined as the (2,4-dinitrophenyl)hydrazone by HPLC after an extraction step. The spiked solutions (see above) were layered with 400 μL of 2,2,4-trimethylpentane and vortexed for 2 h at room temperature to extract the acetaldehyde and butyraldehyde (2,4-dinitrophenyl)-hydrazones into the 2,2,4-trimethylpentane (upper) layer. After vortexing, the upper layer was carefully drawn off the samples for HPLC analysis. Duplicate 200-μL injections of the 2,2,4-trimethylpentane sample were analyzed by reversed phase (C_{18}) HPLC on an Analtech column (4.6 × 250 mm) with isocratic (45:55 water/acetonitrile) elution at ambient temperature and detection at 360 nm (0.010 AUFS). Experimental retention volumes were 10.2 mL for acetaldehyde (2,4-dinitrophenyl)-hydrazone and 21.6 mL for butyraldehyde (2,4-dinitrophenyl)hydrazone. Acetaldehyde was determined by comparing the integrated area of the acetaldehyde (2,4-dinitrophenyl)hydrazone peak to a standard curve for acetaldehyde (2,4-dinitrophenyl)hydrazone prepared in the same manner as above, after correction for extraction efficiency (≥95%) and injection volume as determined by the integrated area of the peak corresponding to butyraldehyde (2,4-dinitrophenyl)hydrazone. Control experiments in the presence of high concentrations of PDC or bovine serum albumin (BSA) were shown to give ≥95% recovery of the acetaldehyde as acetaldehyde (2,4-dinitrophenyl)hydrazone by the method of standard additions.

Solution pH was measured at 25 °C with an Orion Model SA 720 pH meter and Radiometer GK2321C combination electrode standardized at pH 7.00 and 4.00 or 10.00. The value of pD was obtained by adding 0.40 to the observed pH of solutions in D_2O.[14] Protein was determined with bicinchoninic acid[15] with a BSA standard. Kinetic modeling was performed using KINSIM, a kinetic modeling program.[16] Sample recovery from the rapid-quench-flow apparatus and reaction times (t_2) were calibrated with $k_{obsd} = 70\ s^{-1}$ for benzylidenemalanonitrile hydrolysis in 0.5 M KOH.[17]

Results

The kinetics of acetaldehyde formation catalyzed by pyruvamide-activated PDC containing [thiazolium C(2)–L]TDP (L = H or D) at 30 °C in 100 mM sodium MES buffer (pH 6.00) in H_2O containing 10 mM $MgSO_4$, 100 mM pyruvamide, and 12.5 μM sodium pyruvate were followed by HPLC under single-turnover conditions. Typical data are shown in Figure 1 for decarboxylation of 12.5 μM sodium pyruvate to form acetaldehyde catalyzed by >104 μM pyruvamide-activated PDC. Experiments with 33–104 μM pyruvamide-activated PDC containing [thiazole-2-H]TDP were carried out in the same manner as those shown for >104 μM PDC (open circles) with 15–30 time points. Under the reaction conditions of ≥2.6:1 PDC/pyruvate, the quantitative

(7) The term "hydron" refers to the hydrogen cation (L^+) without regard to nuclear mass. The specific names "proton" (^1H) and "deuteron" (^2H) refer to the specific isotopes (Commission on Physical Organic Chemistry, IUPAC. *Pure Appl. Chem.* **1988**, *60*, 1115–1116) and are abbreviated here as $^1H^+$, H; $^2H^+$, D.

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(9) Behforouz, M.; Bolan, J. L.; Flynt, M. S. *J. Org. Chem.* **1985**, *50*, 1186–1189. Allen, C. F. H. *J. Am. Chem. Soc.* **1930**, *52*, 2955–2959. Brady, O. L. *J. Chem. Soc.* **1931**, 756–759.

(10) There was no detectable hydrolysis of TDP (R_f = 0.13) to form thiamin (R_f = 0.35) or thiamin monophosphate (R_f = 0.25), as determined by analysis of 0.5-μL aliquots of the stock TDP solution (pH 6.00) by thin-layer chromatography [silica gel (250-μm) in an inert binder containing a fluorescent indicator] in ethanol/water (60:40) (v/v).

(11) Sieber, M.; König, S. T.; Hübner, G.; Schellenberger, A. *Biomed. Biochem. Acta* **1983**, *4*, 343–349.

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(16) Barshop, B. A.; Wrenn, R. F.; Frieden, C. *Anal. Biochem.* **1983**, *130*, 134–145.

(17) Bernasconi, C. F.; Howard, K. A.; Kanavarioti, A. *J. Am. Chem. Soc.* **1984**, *106*, 6827–6835. See Table II.

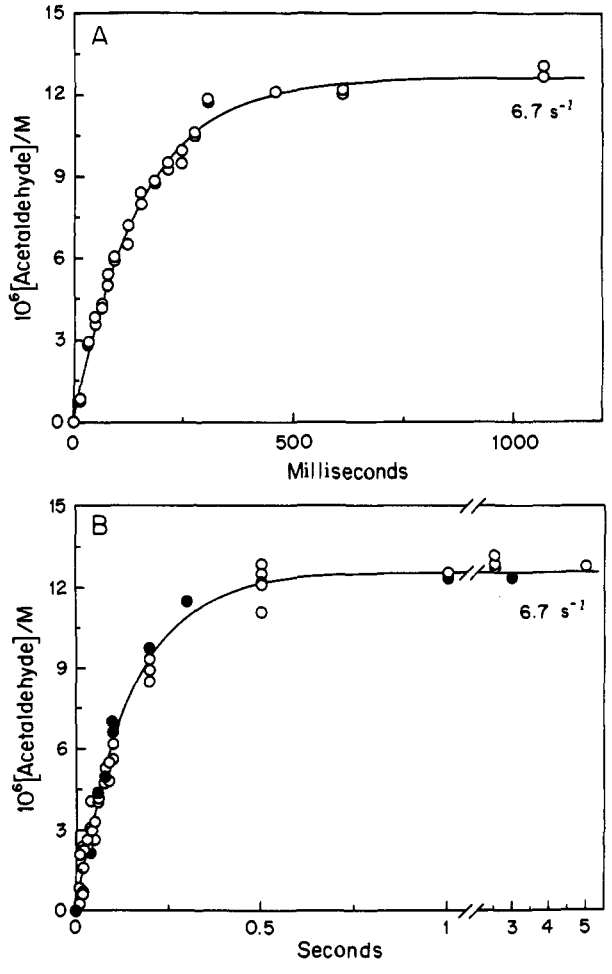


Figure 1. (A) Formation of acetaldehyde catalyzed by 125 μM pyruvamide-activated holo-PDC (30 mg mL^{-1}) containing [thiazole-2-H]TDP at 30 °C in 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM $MgSO_4$, and 12.5 μM sodium pyruvate. The line is drawn for a first-order rate constant of 6.7 s^{-1}. (B) Formation of acetaldehyde catalyzed by 104 μM pyruvamide-activated PDC (25 mg mL^{-1}) containing [thiazole-2-H]TDP (open circles) or [thiazole-2-D]-TDP (solid circles) at 30 °C in 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM $MgSO_4$, and 12.5 μM sodium pyruvate after reconstitution for t_1 = 30 s (see text). The line is drawn for a first-order rate constant of 6.7 s^{-1}. Note the difference in scale between panels A and B.

conversion of pyruvate to form acetaldehyde followed a single exponential and ≥95% of the acetaldehyde was recovered for HPLC analysis as acetaldehyde (2,4-dinitrophenyl)hydrazone. Values of the observed first-order rate constants (k_{obsd}) for acetaldehyde formation catalyzed by 33–150 μM pyruvamide-activated PDC containing [thiazole-2-H]TDP (open circles) or [thiazole-2-D]TDP (solid circles) are summarized in Figure 2. Where multiple determinations of k_{obsd} were made, they agreed within ±7% of the average value.

We found that reconstitution of 6.25–150 μM PDC at 30 °C in 75–100 mM sodium MES buffer (pH 6.00) containing 7.5–10 mM $MgSO_4$ and 10 mM TDP without pyruvate was complete (100% activity was recovered) in ≤19 s, which corresponds to a maximum half-life of 3 s (k_{obsd} ≥ 0.2 s^{-1}) for reconstitution of PDC under these conditions. The presence of 100 mM pyruvamide had no detectable effect on the rate of apo-PDC reconstitution. The fraction of PDC reconstituted to active holo-PDC was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADH to NAD$^+$ upon the addition of sodium pyruvate to a final concentration of 33 mM:[18] plots of

(18) Ullrich, J. *Methods Enzymol.* **1970**, *18A*, 109–115.

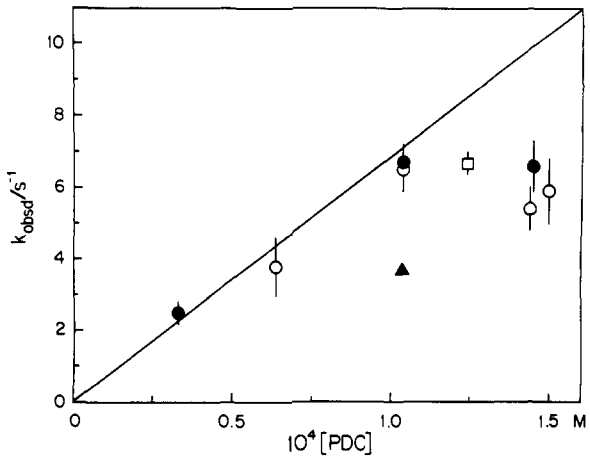


Figure 2. Dependence of the rate constants for acetaldehyde formation catalyzed by pyruvamide-activated PDC on the concentration of PDC in 100 mM sodium MES buffer (pH 6.00) containing 100 mM pyruvamide and 10 mM $MgSO_4$ at 30 °C under single-turnover conditions. PDC was prepared from fresh brewer's yeast (square)[11] or derived from apo-PDC that was reconstituted with Mg^{2+} and [thiazole-2-H]TDP (open circles) or [thiazole-2-D]TDP (solid circles) for 30 s (t_1) before mixing with substrate, marking the onset of t_2 (see text). Values of the first-order rate constants were calculated using nonlinear regression and are expressed as k_{obsd} ± standard error. The line was calculated for [thiazole-2-H]-TDP by numerical integration with rate constants summarized in Scheme II[3c] for the reaction of activated PDC with pyruvate (see text) using the program KINSIM:[16] the rate constant indicated by the triangle was calculated for k_H/k_D = 2 on the addition step (k_3, Scheme II) (see text).

absorbance at 340 nm against time were linear, except for the characteristic lag due to activation of holo-PDC at very early time points in the absence of pyruvamide. The absence of downward curvature in these plots supports the conclusion that reconstitution of PDC was quenched by dilution into assay buffer and no further reconstitution occurred in the presence of substrate under these conditions. Apo-PDC preparations had a specific activity of <0.6 unit mg^{-1} for pyruvate decarboxylation under the above reconstitution conditions without TDP and Mg^{2+}, in the presence of TDP without Mg^{2+}, or in the presence of Mg^{2+} without TDP. A mechanism for reconstitution of apo-PDC with TDP and Mg^{2+} will be presented elsewhere.

Identical values of k_{obsd} were obtained whether the reaction was initiated with holo-PDC or apo-PDC, which provides additional evidence for rapid and complete PDC reconstitution with TDP and Mg^{2+} within 30 s under single-turnover conditions. For example, identical values of k_{obsd} = 6.7 ± 0.4 s^{-1} were obtained for acetaldehyde formation catalyzed by 125 μM pyruvamide-activated holo-PDC containing [thiazole-2-H]TDP (Figure 1A) (t_1 = 0) or 104 μM PDC containing [thiazole-2-H]TDP after reconstitution for t_1 = 30 s (Figure 1B; open circles). These results also support the conclusion that pyruvamide activates PDC completely without a detectable lag time on the millisecond time scale required for single-turnover experiments.[19]

Figures 1B and 2 show that there is little or no primary deuterium kinetic isotope effect, $(k_H/k_D)_{obsd}$ = 1.0 ± 0.1, for C(2)–L abstraction from PDC-bound TDP during catalysis of acetaldehyde formation by 33–150 μM pyruvamide-activated PDC. Figure 1B shows that pyruvamide-activated PDC containing [thiazole-2-D]TDP (solid circles) gave a rate constant of k_{obsd} = 6.7 ± 0.5 s^{-1}, which does not differ significantly from the value of k_{obsd} = 6.7 ± 0.4 s^{-1} for pyruvamide-activated PDC containing [thiazole-2-H]TDP (open circles). Experiments with 33 and 150 μM pyruvamide-activated PDC containing [thiazole-2-D]TDP were carried out as shown for 104 μM pyruvamide-

(19) Hübner, G.; Weidhase, R.; Schellenberger, A. *Eur. J. Biochem.* **1978**, *92*, 175–181. Hübner, G.; König, S.; Schellenberger, A. *Biomed. Biochim. Acta* **1988**, *47*, 9–18.

activated PDC (Figure 1B; solid circles) with 10–15 time points. Single-turnover conditions[20] were required for the measurement of $(k_H/k_D)_{obsd}$ for C(2)-hydron abstraction from PDC-bound TDP because the cofactor TDP is tightly bound to the holoenzyme, TDP dissociates slowly from the holoenzyme, and the fate of the C(2)-hydron during turnover by PDC is not known.[21]

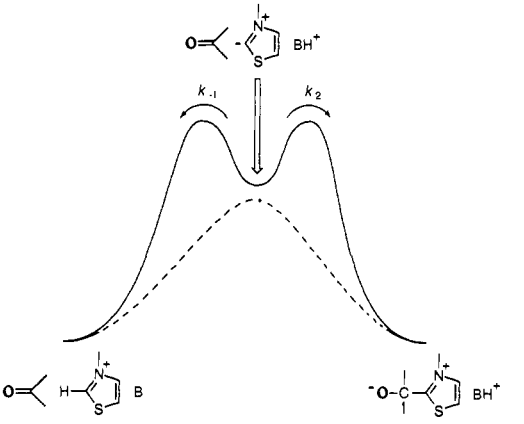
During the 30-s incubation to allow complete reconstitution of apo-PDC, there was ≈10% C(2)–D → H exchange in unbound TDP as determined by ^{1}H NMR.[4] The effect of 10% C(2)–D → H exchange on the value of $(k_H/k_D)_{obsd}$ for C(2)-hydron abstraction from PDC-bound TDP was estimated as described previously.[22a] For 10% C(2)–D → H exchange an isotope effect of 1.10 would be depressed to an observed isotope effect of 1.09, and an isotope effect of 2.0 would be depressed to 1.8. We conclude that 10% C(2)–D → H exchange during reconstitution of apo-PDC would have little or no detectable effect on the observed isotope effect.

Holo-PDC catalyzes 45 ± 3% (n = 2) C(2)–H → D exchange from PDC-bound TDP during the 30-s incubation under the conditions of the single-turnover experiments.[23] Holo-PDC containing [thiazole-2-D]TDP would have catalyzed ≤45% C(2)–D → H exchange in PDC-bound TDP if there was a significant kinetic isotope effect for C(2)–D abstraction under these conditions of the single-turnover experiments in H_2O. The effect of ≤45% C(2)-hydron exchange with solvent on the value of $(k_H/k_D)_{obsd}$ for C(2)-hydron abstraction from PDC-bound TDP was estimated as described previously.[22b] Modeling shows that for this extent of C(2)-hydron exchange a value of $(k_H/k_D)_{obsd} \geq 1.2$ would give a significant deviation from a simple first-order plot of the single-turnover data (Figure 1). We conclude that the observation of first-order kinetics for acetaldehyde formation means that a value of $(k_H/k_D)_{obsd} \geq 1.2$ would have been detectable in these experiments even with this extent of C(2)-hydron exchange.

Discussion

Comparison of Modeled and Experimental Results. The observed first-order rate constants for acetaldehyde formation by pyruvamide-activated PDC increase over the range k_{obsd} = 2.5–6.7 s^{-1} with increasing PDC concentration in the range 33–104 μM and are independent of PDC concentration in the range 104–150 μM with a value of k_{obsd} = 6.7 s^{-1} (Figure 2). The values of k_{obsd} obtained at 33 μM < [PDC] < 104 μM agree with values calculated using the microscopic rate constants and kinetic mechanism summarized in Scheme II[3c] for the reaction of activated PDC (^{c}EA) with pyruvate (S) (Figure 2; solid line). The agreement between the observed and calculated rate constants provides additional evidence that incubation of PDC with pyruvamide, before addition of substrate and the onset of t_2, rapidly and completely activates PDC:[19] this means that activation of PDC is not rate limiting under these experimental conditions.

Scheme III



These results also support the idea that microscopic rate constants for an enzymatic reaction can be calculated from kinetic isotope effects on the enzymatic reaction and related nonenzymatic model reactions,[3c,24] support the conclusion that reconstitution of apo-PDC with TDP and Mg^{2+} is complete within 30 s under single-turnover conditions, and provide evidence that reliable values of k_{obsd} can be obtained with the rapid-quench apparatus under single-turnover conditions at relatively high [PDC].

The value of k_{obsd} = 6.7 s^{-1} at [PDC] > 104 μM, where acetaldehyde formation becomes zero-order in [PDC], is less than k_{cat} = 45–80 s^{-1} for pyruvamide-activated PDC (assuming four occupied and catalytically active TDP binding sites),[3c] and deviates negatively from the values of k_{obsd} calculated from Scheme II under these reaction conditions. The observation of a single exponential over the range 33–150 μM PDC (Figure 1) and the decreasing concentration dependence of the values of k_{obsd} at >104 μM PDC (Figure 2) suggests either a change in rate-limiting step[20b] or some type of complex formation involving the reactants at high [PDC].

C(2)-Proton Transfer from TDP and the Nature of the Addition Reaction Transition State. A pK_a value of ≤14 is required for PDC-bound TDP if the ylide exists as a discrete intermediate in a stepwise enzymatic addition mechanism (Scheme III; solid line).[4] Values of pK_a for C(2)–H in several 3-substituted 4-methylthiazolium ions, including thiamin, were found to be in the range pK_a = 17–19 in H_2O; this pK_a range for C(2)–H in thiazolium ions indicates that their proton-transfer reactions are thermodynamically unfavorable in aqueous solution.[4–6] Therefore either PDC changes the relative thermodynamic stabilities of TDP and the ylide or PDC provides a preassociation or a concerted pathway for the addition step (Scheme II; step 3) that avoids such an unstable carbanion intermediate.[4,25] Analysis of primary kinetic isotope effects for enzyme-catalyzed C(2)-hydron abstraction can provide evidence for sequential transition states in the addition step ("kinetic complexity")[26] and provide details about the relative properties of sequential transition states.[26c]

There is little or no observed primary kinetic isotope effect, $(k_H/k_D)_{obsd} \leq 1.2$, for C(2)–L abstraction from PDC-bound TDP by 33–150 μM pyruvamide-activated PDC (Figure 2). Numerical integration with microscopic rate constants summarized in Scheme II[3c] using the program KINSIM[16] suggests that an isotope effect

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(23) Holo-PDC containing [thiazole-2-H]TDP in reaction buffer was lyophilized and dissolved in D_2O at 30 °C, and C(2)–H → D exchange was quenched after 30 s with DCl. The quenched sample was centrifuged, and [thiazole-2-L]TDP in the supernate was analyzed by ^{1}H NMR. The extent of PDC-catalyzed C(2)-hydron exchange was calculated with $1 - A_2^+/A_2^-$, where A_2 is the integrated area of the C(2)–H signal ($\delta \approx 9.3$ ppm), normalized to the integrated area for the C(6′)–H signal ($\delta \approx 7.6$ ppm) (as nonexchanging internal standard), in the presence (+) or absence (–) of PDC. No loss of PDC activity was observed after lyophilization. No free TDP, which was shown to exchange relatively slowly (see above), was detectable in holo-PDC solutions after treatment by size-exclusion chromatography before or after lyophilization. On the basis of these control experiments we conclude that holo-PDC catalyzes *incomplete* C(2)-hydron exchange from PDC-bound TDP in 30 s.

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of ≤2.0 on the addition step (k_3, Scheme II) would have been detectable under the conditions of the single-turnover experiments (Figure 2; triangle). The small isotope effect suggests that the C(2)-hydron has not lost a significant amount of zero-point energy because of being "in flight" in the transition state. Isotope effects for the action of PDC on pyruvate, [1-^{13}C]pyruvate, [2-^{13}C]-pyruvate, and [3-D_3]pyruvate provide evidence against simple explanations that pyruvate binding or acetaldehyde release are completely rate limiting.[3c] Incomplete C(2)-hydron exchange from PDC-bound TDP in 30 s provides evidence against facile C(2)–D → H exchange at the enzyme active site, significantly depressing the actual value of $(k_H/k_D)_{obsd}$ because of washout of the isotopic label before turnover. There are several other possible reasons for the small isotope effect:

(1) Small isotope effects can result from rate-limiting conformational-reorganization events preceding addition of TDP at the substrate carbonyl group. The magnitudes of complex isotope effects for the action of PDC on pyruvate, [2-^{13}C]pyruvate, and [3-D_3]pyruvate suggest little or no progression of nucleophilic attack at the substrate keto group within the addition step.[3c] This would be consistent with either conformational-reorganization events preceding addition of TDP to the substrate carbonyl group or an "early" transition state involving a relatively small amount of C–C bond formation between the carbonyl group and the C(2) position of TDP (Scheme III; broken line or $k_{-1} > k_2$). An early structure for the carbonyl-addition transition state would be consistent with the relatively high basicity of the ylide derived from TDP[3c,4] and evidence for a significant amount of negative charge development on the leaving C(2) carbon atom in the transition state for cleavage of 2-(1-hydroxyethyl)-3-*R*-4-methylthiazolium ions.[25]

(2) The observed isotope effect is expected to approach the equilibrium isotope effect for C(2)-hydron abstraction if PDC uses a stepwise mechanism (Scheme III; solid line) involving rapid equilibrium formation of the ylide before C–C bond formation ($k_{-1} > k_2$).[26] The fractionation factor is 0.97 ± 0.05 for nonenzymatic C(2)–H → D exchange from TDP in aqueous solution.[4,27]

(3) Small isotope effects can result from proton movement coupled to heavy atom motion in the transition state of a concerted, coupled mechanism (Scheme III; broken line).[28] Coupling between proton donation and leaving group expulsion for aldol-type reactions involving thiamin and acetaldehyde in aqueous solution suggests that catalysis by PDC could involve assistance to C(2)-proton removal by interaction with the electrophile and assistance to carbon–carbon bond formation by a significant amount of C(2)-proton transfer, in the transition state of a concerted mechanism.[25] Catalysis of C(2)-hydron exchange by PDC does not exclude a concerted mechanism for the addition step: a lower pK_a for C(2)–H in PDC-bound TDP, besides stabilizing the ylide for a stepwise mechanism,[4] would facilitate C(2)–H abstraction in a concerted mechanism.

(4) Small isotope effects can occur if the donor, the hydron, and the acceptor atom are not in a straight line in the transition state for enzyme-catalyzed proton transfer to form the ylide as a discrete enzyme-bound intermediate (Scheme III; $k_{-1} < k_2$ or $k_{-1} \approx k_2$).[29] Geometric constraints could implicate nonlinear hydron transfer in proposed enzymatic mechanisms[30] invoking intramolecular general base catalysis of C(2)-hydron abstraction from enzyme-bound TDP by the exocyclic 4′-imino group of N(1′)-protonated TDP. No evidence for an intramolecular mechanism involving the 4′-imino group was obtained for *nonenzymatic* C(2)-proton abstraction from *N*(1′)-methylthiamin.[4]

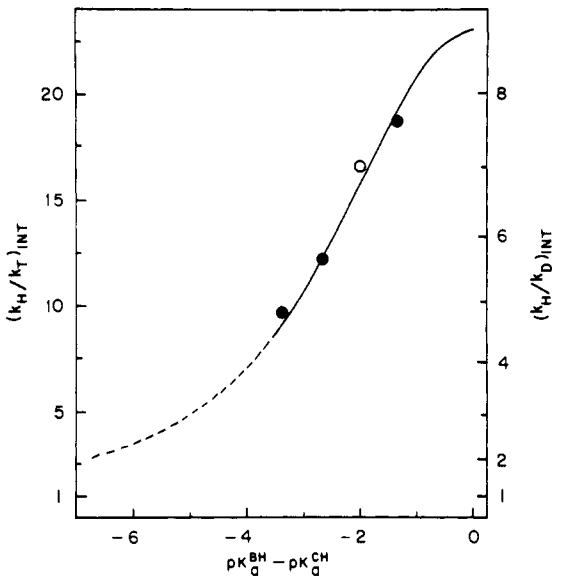

Figure 3. Dependence of the intrinsic primary kinetic isotope effect for C(2)-hydron exchange from 3-R-4-methylthiazolium ions (solid circles) and N(1′)-protonated thiamin (open circle) on ΔpK_a in H_2O at 30 °C and I = 2.0 M (NaCl). The lines were calculated from a modified Marcus equation[32] that is applicable over such a large change in ΔpK_a with an intrinsic barrier of 1.4 kcal mol^{-1}, a work term of 3.3 kcal mol^{-1}, a value of $(k_H/k_T)_{int}$ = 23 at ΔpK_a = 0, and the relationship $\log(k_H/k_T) = 1.44 \log(k_H/k_D)$;[33] the data are for catalysis by hydroxide ion.[6]

(5) Asymmetry in the transition state for proton transfer to form the ylide as a discrete intermediate (Scheme III; $k_{-1} < k_2$ or $k_{-1} \approx k_2$) can result in suppression of the primary kinetic isotope effect.[31] Figure 3 shows the increase in the value of the intrinsic primary kinetic isotope effect, $(k_H/k_T)_{int}$ and $(k_H/k_D)_{int}$, for C(2)-hydron transfer from 3-substituted thiazolium ions as the difference ΔpK_a = pK_a^{BH} − p$K_a^{C(2)H}$ approaches zero for nonenzymatic C(2)-hydron abstraction in aqueous solution. The abrupt change in the value of $(k_H/k_L)_{int}$ for C(2)–L abstraction as the acidity of the carbon acid is changed is much sharper than that usually observed for hydron transfer to or from carbon[34] and is consistent with a small barrier for C(2)–L abstraction that is similar to that for the electronegative atoms of "normal" acids.[6] A small barrier has a small curvature that allows the position of the transition state to slide easily over the energy surface as the energy of the hydron donor or the base is changed, which gives a change in transition-state structure and in the observed isotope effect as described by Melander and Westheimer.[31]

The abrupt change in the value of $(k_H/k_L)_{int}$ as ΔpK_a approaches zero suggests that values of $(k_H/k_L)_{int}$ significantly greater than 1 will be obtained only when $-7 \le \Delta pK_a \le 7$ if geometrical constraints are similar in the transition state for C(2)-hydron transfer from free and enzyme-bound TDP. The broken line in Figure 3 is outside the experimental data, which means that the limits of $-7 \le \Delta pK_a \le 7$ are approximate. We estimate that the ΔpK_a limits are valid to within ±1. A narrower ΔpK_a range is expected if the transition state for C(2)-hydron transfer from enzyme-bound TDP is nonlinear: the value of $(k_H/k_L)_{int}$ for a given value of ΔpK_a would be less than the value predicted from Figure 3. It is not possible to estimate a limiting pK_a value for C(2)–H in enzyme-bound TDP from the limits $-7 \le \Delta pK_a \le 7$

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without knowing the pK_a value of the catalytic base in the enzyme–substrate–TDP complex.

We assume that PDC does not significantly change the minimal intrinsic barrier (1.3 ± 0.3 kcal mol^{-1})[6,35] and, consequently, the dependence of $(k_H/k_L)_{int}$ for C(2)-proton transfer on ΔpK_a (Figure 3). The intrinsic barrier for hydrogen transfer is independent of changes in the equilibrium constant for reactions catalyzed by several dehydrogenases, which supports this assumption.[36] Similar intrinsic barriers are expected for C(2)-hydron abstraction from free TDP in aqueous solution and TDP bound to the nonpolar binding site on yeast PDC:[37] the intrinsic barrier for proton transfer to and from carbon generally decreases with, or is independent of, a change from an aqueous to a less polar, aprotic solvent.[38] There is no obvious advantage for an enzyme to increase the intrinsic barrier for C(2)-proton abstraction, and it is unlikely that the already small intrinsic barrier could be decreased significantly.

Conclusions. (1) The small intrinsic barrier for C(2)-hydron exchange from thiazolium ions, which gives an unusually large change in transition-state structure[6] and in the observed primary kinetic isotope effect as the acidity of the carbon acid or catalytic base is changed, likely limits the range of measurable primary kinetic isotope effects in TDP-dependent enzymes to cases where $-7 \leq \Delta pK_a (= pK_a^{BH} - pK_a^{C(2)H}) \leq 7$. (2) Catalysis of incomplete C(2)-hydron exchange from PDC-bound TDP does not distinguish between a small decrease in the pK_a value for C(2)–H and a large decrease in this pK_a with only a small amount of exchange of the hydron abstracted from C(2) with solvent hydrons: C(2)-hydron removal occurs at the maximum possible rate for a given equilibrium constant.[4] (3) The primary kinetic isotope effect, $(k_H/k_D)_{obsd} \leq 1.2$, for C(2)-hydron abstraction from PDC-bound TDP is inconsistent with an enzymatic mechanism involving rate-limiting C(2)–H transfer between TDP and a catalytic base with $-7 \leq \Delta pK_a \leq 7$ to form a discrete ylide intermediate (Scheme III; $k_{-1} < k_2$ or $k_{-1} \approx k_2$). (4) The absence of a primary kinetic isotope effect for C(2)-hydron abstraction from PDC-bound TDP is consistent with a concerted enzymatic mechanism, a stepwise enzymatic mechanism involving rapid equilibrium formation of the ylide as a discrete intermediate followed by rate-limiting C–C bond formation, or a rate-limiting conformational change preceding addition of TDP to the substrate carbonyl group.

(35) The intrinsic barrier is the kinetic barrier when there is no thermodynamic driving force for a reaction.

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Acknowledgment. The NMR studies were performed in the Biophysics NMR Facility at Johns Hopkins University, which was established by a grant from the National Institutes of Health (GM 27512) and a Biomedical Shared Instrumentation Grant (1S10-RR06261-0). We are grateful to a reviewer for helpful comments and for suggesting the experiment to determine whether PDC catalyzes C(2)-hydron exchange from PDC-bound TDP. We thank the Anheuser-Busch Brewing Co., Newark, NJ, for the continuous supply of fresh brewer's yeast.