Distribution of the Thiamin Diphosphate C(2)-Proton during Catalysis of Acetaldehyde Formation by Brewers' Yeast Pyruvate Decarboxylase†

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ABSTRACT: The distribution of tritium derived from enzyme-bound [thiazole-2-T]thiamin diphosphate (TDP) during the reaction of pyruvate to form acetaldehyde catalyzed by pyruvate decarboxylase isozymes (PDC; EC 4.1.1.1) from *Saccharomyces carlsbergensis* was determined under single-turnover conditions ([E] > [S]) in the presence of the nonsubstrate allosteric effector pyruvamide. The specific radioactivity of the [1-L]acetaldehyde product and solvent ([L]H_2O) was 43 ± 4% and 54 ± 2%, respectively, of the initial specific radioactivity of PDC-bound [thiazole-2-T]TDP and was independent of the extent of the single-turnover reaction. There is little (≤3%) or no return of the abstracted C(2)-hydron to the C(2) position of PDC-bound TDP. This provides evidence that the abstracted C(2)-hydron is involved in the specific protonation of the C(α) position of the PDC-bound intermediate 2-(1-hydroxyethyl)thiamin diphosphate (HETDP), which is cleaved to form [1-L]acetaldehyde and PDC-bound [thiazole-2-H]TDP. The partial exchange of C(2)-derived tritium into solvent requires that (1) hydron transfer from C(2) occurs to a catalytic base in which the conjugate catalytic acid is partially shielded from hydron exchange with the solvent, (2) the conjugate catalytic acid transfers the C(2)-derived hydron to the C(α) position of HETDP, and (3) hydron transfer to C(2) to regenerate the coenzyme occurs either from solvent directly or from a second catalytic acid of the enzyme that undergoes rapid hydron exchange with the solvent. The observed rate constant k_{obsd} = 1.4 min^{-1} for C(2)-hydron exchange in PDC-bound TDP in the absence of substrate corresponds to a pK_a value for C(2)-H at the active site of 17.5.

Pyruvate decarboxylase (PDC)[1] (2-oxo-acid carboxy-lyase; EC 4.1.1.1) is a thiamin diphosphate (TDP, **1a**) dependent enzyme that catalyzes the irreversible nonoxidative decarboxylation of pyruvate to form acetaldehyde (Scheme 1) (Alvarez et al., 1991, 1995; Crane et al., 1993). PDC also catalyzes an aldol-type condensation reaction between two molecules of acetaldehyde to form the α-ketol acetoin (Stivers & Washabaugh, 1993). The C(2)-ylide **2** derived from TDP (**1a**) and the C(α)-carbanion/enamine **3** derived from 2-(1-hydroxyethyl)thiamin diphosphate (HETDP, **4a**) have been implicated in reactions catalyzed by several TDP-dependent enzymes (Kluger, 1992). We are interested in the factor(s) that contribute to the kinetic barrier(s) and how PDC catalyzes proton transfer to and from the C(2) position of TDP and the C(α) position of HETDP.

The goal of the work reported here was to determine the distribution of the C(2)-proton derived from TDP and define the source of catalytic protons for protonation of the C(2) position in TDP and the C(α) position in HETDP. In this paper we describe evidence that these proton-transfer reactions require at least two catalytic groups. The distribution of tritium derived from the C(2) position of PDC-bound [thiazole-2-T]TDP provides evidence that the abstracted C(2)-hydron[2] is involved in the specific protonation of the C(α) position of the PDC-bound intermediate HETDP. Partial exchange of C(2)-derived tritium into solvent requires that hydron transfer from C(2) occurs to a catalytic base in which the conjugate catalytic acid is partially shielded from hydron exchange with the solvent, and the conjugate catalytic acid transfers the C(2)-derived hydron to the C(α) position of HETDP. Hydron transfer to C(2) to regenerate the coenzyme occurs either from solvent directly or from a second catalytic acid of the enzyme that undergoes rapid hydron exchange with the solvent. We confirm and extend the previous conclusion (Crane et al., 1993) that the rate of C(2)-hydron transfer from PDC-bound TDP in the absence of substrate is slow−$10^{3.5}$-fold slower than the rate predicted on the basis of k_{cat} for acetaldehyde formation from pyruvate and only 4-fold faster than for C(2)-hydron transfer from free TDP in aqueous solution.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of analytical or reagent grade and were used without further purification unless otherwise indicated. All water was prepared on a four-bowl Milli-Q water system including an Organex-Q cartridge (Millipore). Benzylidenemalanonitrile, pyruvamide, and the (2,4-dinitrophenyl)hydrazones of acetaldehyde and butyraldehyde (HPLC standards) were prepared as described previously (Crane et al., 1993). 1-(Carboxymethyl)pyridinium chloride hydrazide (Girard's reagent P) was purchased from

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[1] Abbreviations: PDC, pyruvate decarboxylase; TDP, thiamin diphosphate; HETDP, 2-(1-hydroxyethyl)thiamin diphosphate; Girard's reagent P, 1-(carboxymethyl)pyridinium chloride hydrazide; MES, 2-(*N*-morpholino)ethanesulfonate; Me_2SO, dimethyl sulfoxide; ADH, alcohol dehydrogenase; NADH (NAD$^+$), reduced (oxidized) nicotinamide adenine dinucleotide; U, enzyme activity unit.

[2] The term "hydron" refers to the hydrogen cation (L^+) without regard to nuclear mass. The specific names "proton" (^1H), "deuteron" (^2H), and "triton" (^3H) refer to the specific isotopes (IUPAC Commission on Physical Organic Chemistry, 1988) and are abbreviated here as $^1H^+$, H; $^2H^+$, D; and $^3H^+$, T.

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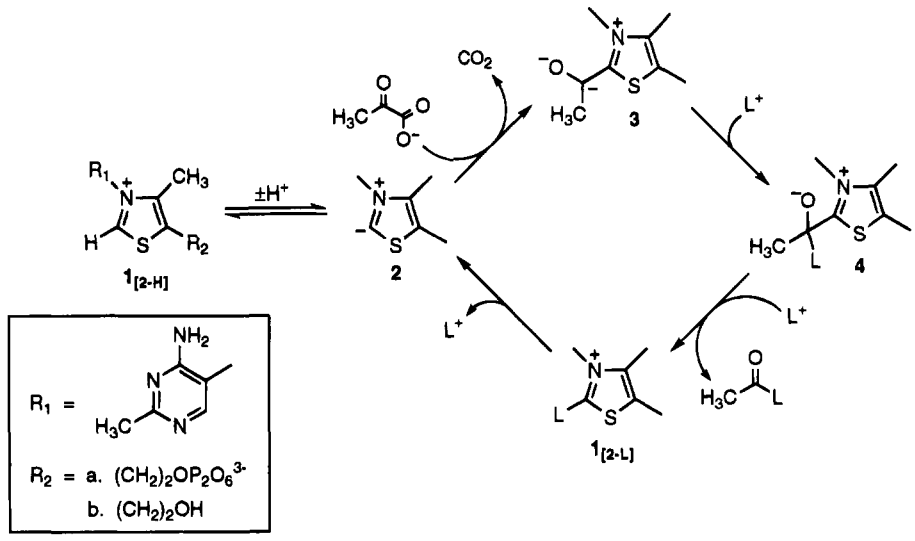
Scheme 1



Aldrich. N(1′)-protonated [thiazole-2-T]TDP (pK_a = 5.1) (Washabaugh & Jencks, 1988, 1989a) was prepared from N(1′)-protonated [thiazole-2-H]TDP (9.2 mg; 20 μmol) by exchange with 100 μL of [^{3}H]H_2O (1.8 Ci mol^{-1}) in H_2O for 24 h at 25 °C and pH 5−6 (the pH was adjusted with KOH). The solvent was removed by evaporation under a water aspirator vacuum in a Savant Speed-Vac centrifugal concentrator. The [thiazole-2-T]TDP (1.8 Ci mol^{-1}) was dissolved in 100 μL of 1 M HCl and the solvent, which contained exchanged tritium as [^{3}H]H_2O and aqueous HCl, was removed *in vacuo*; the N(1′)-protonated [thiazole-2-T]-TDP was dissolved in 50 mM HCl and stored at −20 °C until use (≤24 h). Parallel experiments in deuterium oxide and ^{1}H NMR examination of the exchanged TDP showed that isotopic labeling under these conditions occurs exclusively by exchange at C(2)-H.[3] Brewers' yeast PDC holoenzyme (Sieber et al., 1983) and apo-PDC (Gubler & Wittorf, 1970) were prepared as described previously. "Unresolved" PDC containing the α_4 and $\alpha'_2\beta_2$ isozymes was used in the experiments reported here (Kuo et al., 1986). One enzyme activity unit represents the conversion of 1 μmol of pyruvate to acetaldehyde per minute at 30 °C (Ullrich, 1970).

General Methods. All operations with volatile radioactivity were performed in a hood. Protein was determined with bicinchoninic acid (Smith et al., 1985) with a bovine serum albumin standard. Melting points were determined on an Electrothermal Model IA9100 digital melting point apparatus and are uncorrected. Solution pH was measured at 30 °C with an Orion Model SA 720 pH meter and Radiometer GK2321C combination electrode standardized at pH 6.99 and 4.01 or 9.96. The electrode was free of anomalous ionic strength effects (Illingworth, 1981). Kinetic modeling was performed using KINSIM, a kinetic modeling program (Barshop et al., 1983). Sample recovery from the rapid-quench-flow apparatus and reaction times were calibrated with k_{obsd} = 35 s^{-1} for benzylidenemalanonitrile hydrolysis in 0.25 M KOH (Bernasconi et al., 1984). ^{1}H NMR spectra in mixed H_2O-D_2O solutions were recorded on a Bruker AMX-300 300 MHz NMR spectrometer. Radioactivity was measured using a Beckman LS 7500 liquid scintillation counter.

Activation of PDC. Rapid activation of apo-PDC was performed with a rapid-quench-flow apparatus operating in a "four-syringe" mode and thermostated at 30.0 ± 0.2 °C. "Reaction buffer" refers to 100 mM sodium MES buffer (pH 6.00), 10 mM $MgSO_4$, and 100 mM pyruvamide in H_2O. One sample loop was loaded with 35 μL of 75 μM (18 mg mL^{-1}) apo-PDC in 300 mM sodium MES buffer (pH 6.64), 30 mM $MgSO_4$, and 300 mM pyruvamide. The other sample loop contained 35 μL of 1 mM TDP in 50 mM HCl. The left and right drive syringes were filled with reaction buffer, and the middle drive syringe was filled with H_2O. The activation reaction was initiated by mixing the contents of the sample loops to obtain a solution containing 37.5 μM apo-PDC, 150 mM sodium MES buffer (pH 6.00), 15 mM $MgSO_4$, 150 mM pyruvamide, and 0.5 mM TDP that was allowed to react for a time t_1 ($0 \leq t_1 \leq 180$ s) before the reaction solution (255 μL) was quenched by dilution into 6.4 mL of reaction buffer. The fraction of apo-PDC activated to form active holo-PDC at each time point (t_1) was determined with yeast alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase; EC 1.1.1.1) and NADH 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 (Ullrich, 1970). Plots of absorbance at 340 nm against time were linear. The absence of downward curvature in these plots supports the conclusion that activation of PDC was quenched by dilution into assay buffer and no further activation occurred in the presence of substrate under these conditions.

Determination of SA^P/SA^{TDP}. The distribution, SA^P/SA^{TDP}, of tritium transferred from PDC-bound [thiazole-2-T]TDP (SA^{TDP}) to [1-L]acetaldehyde or [L]H_2O (SA^P) in rapid-quench kinetic experiments was determined from the ratio of the specific radioactivities of the [1-L]acetaldehyde product or solvent and the PDC-bound [thiazole-2-T]TDP during a single turnover. The left and right drive syringes in the rapid-quench-flow apparatus (operating in a four-syringe mode at 30.0 ± 0.2 °C) were filled with reaction

[3] Analysis of the exchanged TDP by reversed-phase (C_{18}) HPLC on a Whatman column (4.6 × 250 mm) with isocratic [200 mM aqueous sodium phosphate buffer (pH 4.2) containing 1% (v/v) acetonitrile] elution at ambient temperature and detection at 245 nm (0.5 AUFS) showed no significant (≤1%) breakdown to form thiamin monophosphate, thiamin, or oxythiamin species under these exchange and storage conditions. The experimental retention volumes were 3.9 mL for TDP (ϵ_{245} = 14.9 × 10^3 M^{-1} cm^{-1}), 4.8 mL for thiamin monophosphate, 9.7 mL for thiamin, and 16.9 mL for oxythiamin.

buffer. A typical experiment was initiated by mixing 35 μL of 1.0 mM [thiazole-2-T]TDP (1.8 Ci mol^{-1}) in 50 mM HCl contained in one sample loop with 35 μL of 420 μM apo-PDC (100 mg mL^{-1}) in 300 mM sodium MES buffer (pH 6.64), 300 mM pyruvamide, and 30 mM $MgSO_4$ contained in the other sample loop, giving final concentrations of 210 μM apo-PDC, 0.5 mM [thiazole-2-T]TDP, 150 mM pyruvamide, and 15 mM $MgSO_4$ in 150 mM sodium MES buffer (pH 6.00). The apo-PDC/cofactor solution was allowed to react for 3 s (t_1) to obtain 80 μM holo-PDC (320 μM E active sites)—38% activated apo-PDC. The decarboxylation reaction was initiated by mixing the contents of the sample loops with 35 μL of 75 μM sodium pyruvate contained in the middle drive syringe to obtain a solution containing 50 μM holo-PDC (200 μM E active sites), 25 μM sodium pyruvate, 0.3 mM [thiazole-2-T]TDP, 100 mM pyruvamide, and 10 mM $MgSO_4$ in 100 mM sodium MES buffer (pH 6.00). The holo-PDC/pyruvate solution was allowed to react for a time t_2 ($0.03 \leq t_2 \leq 2$ s) in the exit line. The reaction was acid-quenched by collecting the 250-μL reaction solution (including 145 μL from the left and right drive syringes) in a 1.5-mL microcentrifuge tube containing 50 μL of 4 M HCl. Control reactions for all experiments were performed in the absence of pyruvate (the middle drive syringe contained H_2O) to determine background radioactivity released into the solvent. The 300-μL quenched samples were centrifuged for $\geq$20 min at 16000g. [1-T]Acetaldehyde produced at each time point t_2 ($\leq$0.14 nmol) was determined as the (2,4-dinitrophenyl)hydrazone by HPLC after an extraction step as described previously (Crane & Washabaugh, 1993). Total [^{3}H]H_2O produced at each time point t_2 ($\leq$0.9 Ci mol^{-1}) was determined as described previously (Stivers & Washabaugh, 1993) after treatment with Girard's reagent P to convert volatile carbonyl compounds to nonvolatile hydrazones (Mitchell & Birnboim, 1977). Background [^{3}H]H_2O (0.12 Ci mol^{-1}; 6.4% of the [thiazole-2-T]TDP) due to C(2)-T$\rightarrow$H exchange in free and PDC-bound [thiazole-2-T]TDP during the activation step (t_1 = 3 s) in the absence of substrate was subtracted from the total amount of [^{3}H]H_2O produced at each time point. Accordingly, the value of SA^{TDP} = 1.7 Ci mol^{-1} was calculated by multiplying the initial specific radioactivity of [thiazole-2-T]TDP (1.8 Ci mol^{-1}) by 93.6% (= 100% − 6.4%).

Kinetics. Rate constants for C(2)-T$\rightarrow$H exchange in free TDP were determined by measuring nonvolatile, unexchanged tritium remaining in [thiazole-2-T]TDP at 30 °C as described previously (Washabaugh & Jencks, 1989a). Rate constants for C(2)-H$\rightarrow$T exchange in pyruvamide-activated PDC-bound [thiazole-2-H]TDP were determined with a rapid-quench-flow apparatus operating in a "three-syringe" mode and thermostated at 30.0 ± 0.2 °C. The left and right drive syringes in the rapid-quench-flow apparatus were filled with reaction buffer and the middle (quench) syringe was filled with 1 M HCl. One sample loop was loaded with 35 μL of [^{3}H]H_2O (0.22 Ci mol^{-1}) in H_2O. The other sample loop contained 35 μL of 80 μM holo-PDC in 200 mM sodium MES buffer (pH 6.00) containing 200 mM pyruvamide and 20 mM $MgSO_4$. The exchange reaction was initiated by mixing the contents of the sample loops to obtain a solution that was allowed to react for a time t ($5 < t < 200$ s) before the reaction was acid-quenched. The final concentrations were 40 μM holo-PDC (160 μM E active sites), 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, and 10 mM $MgSO_4$ in [^{3}H]H_2O (0.11 Ci mol^{-1}). The 165−275-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 $\geq$20 min at 16000g. [Thiazole-2-T]TDP was isolated using spin column cation-exchange chromatography as described previously (Washabaugh & Collins, 1986; Harris & Washabaugh, 1995). All radioactivity in the TDP-containing eluates was shown to be contained in TDP-containing fractions by reversed-phase (C_{18}) HPLC; TDP was determined spectrophotometrically by its absorbance at 245 nm.[3] Fractions containing TDP were counted for at least 10^5 counts with automatic quench control. A 500-μL aliquot of the TDP-containing eluate typically gave $\leq$600 DPM after subtraction of background counts ($\leq$50 DPM). [Thiazole-2-T]TDP specific radioactivity (SA^{TDP}; 0.02−0.11 Ci mol^{-1}) was calculated by dividing the total radioactivity recovered in the [thiazole-2-T]TDP-containing eluate (after subtraction of background counts) by the amount of TDP in the aliquot.

Pseudo-first-order rate constants were obtained from semilogarithmic plots of ($D_t - D_\infty$) against time (D = DPM) and the relationship $k_{obsd} = 0.693/t_{1/2}$. These plots were linear for $> 3t_{1/2}$ with $\geq$10 time points. When duplicate determinations of k_{obsd} were made, they agreed within ±5% of the average value. Values are reported as the mean ± the standard error of the mean.

RESULTS

Determination of the distribution (product, solvent, or that retrieved by the coenzyme) of tritium derived from PDC-bound [thiazole-2-T]TDP requires pre-steady-state kinetics to follow only a single turnover of the enzyme. Single-turnover studies are required because the cofactor TDP is tightly bound to the holoenzyme and dissociates slowly from the holoenzyme [see, for example, Vaccaro et al. (1995)]: this represents a "high commitment to catalysis" (Northrop, 1982). Under single-turnover conditions the enzyme is in excess of substrate so that only a fraction of the active sites undergo turnover. Control experiments under single-turnover conditions were required to (1) confirm the conclusion that PDC does not catalyze facile C(2)-hydron exchange between solvent and PDC-bound TDP (Crane et al., 1993), (2) establish and minimize the amount of background release of [^{3}H]H_2O produced by enzymic and nonenzymic C(2)-T$\rightarrow$H exchange in the absence of substrate for subtraction from the total amount of volatile [^{3}H]H_2O produced in the presence of substrate, and (3) maximize the amount of holo-PDC produced during activation of apo-PDC·Mg^{2+} with [thiazole-2-T]TDP (Vaccaro et al., 1995) before addition of substrate.

Typical data are shown in Figure 1 for C(2)-T$\rightarrow$H exchange in free [thiazole-2-T]TDP and C(2)-H$\rightarrow$T exchange in pyruvamide-activated PDC-bound [thiazole-2-H]TDP in aqueous solution at 30 °C in 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, and 10 mM $MgSO_4$. Pyruvamide does not compete with pyruvate at the active site but binds elsewhere on the protein as an allosteric effector (Hübner et al., 1978). Rate constants, k_{obsd}, were determined by measuring nonvolatile tritium in [thiazole-2-L]TDP (L = H or T) under pseudo-first-order conditions. The value of k_{obsd} = 0.32 min^{-1} for C(2)-T$\rightarrow$H exchange from free [thiazole-2-T]TDP in aqueous solution agrees with a previously determined value at ionic strength 0.22 M (Washabaugh & Jencks, 1989b). Values of k_{obsd} for C(2)-hydron exchange from thiamin do not change significantly

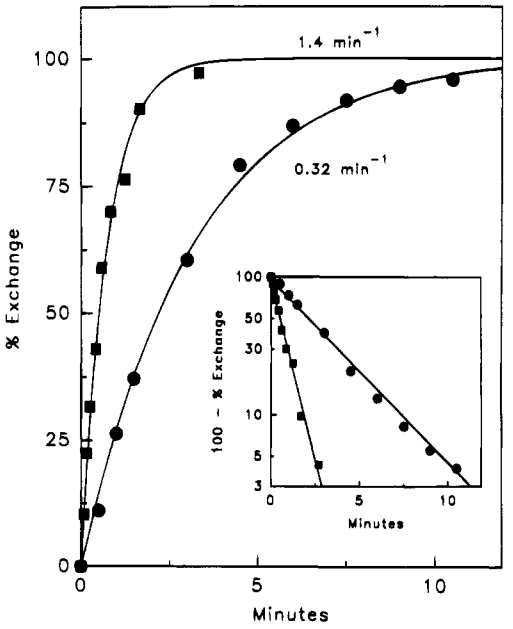


FIGURE 1: C(2)-T→H exchange in 0.27 mM free [thiazole-2-T]-TDP (●) and C(2)-H→T exchange in 40 μM PDC-bound [thiazole-2-H]TDP (■) at 30 °C in H_2O (●) or [^{3}H]H_2O (0.11 Ci mol^{-1}) (■) containing 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, and 10 mM $MgSO_4$. The lines are drawn for first-order rate constants (inset) of 0.32 min^{-1} (●) and 1.4 min^{-1} (■), respectively.

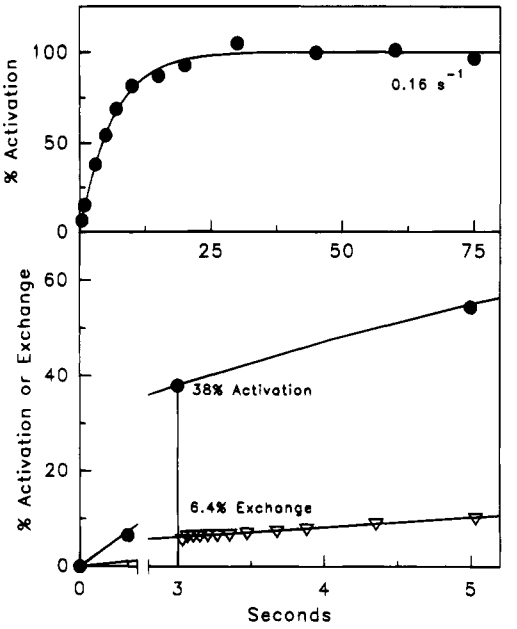


FIGURE 2: Upper panel: activation of 37.5 μM apo-PDC·Mg^{2+} (9.0 mg mL^{-1}) with 0.5 mM [thiazole-2-T]TDP (1.8 Ci mol^{-1}) at 30 °C in 150 mM sodium MES buffer (pH 6.00), 150 mM pyruvamide, and 15 mM $MgSO_4$. The apoenzyme was preincubated with $MgSO_4$, and the activation reaction was initiated by the addition of [thiazole-2-T]TDP (see text). The specific activity of 100% activated PDC was 50 U mg^{-1}. The line is drawn for a first-order rate constant of 0.16 s^{-1}. Lower panel: replot showing 38% activation of apo-PDC·Mg^{2+} with [thiazole-2-T]TDP (●) in 3 s under conditions where 6.4% C(2)-T→H exchange occurred in free and PDC-bound [thiazole-2-T]TDP (▽) during the activation reaction. C(2)-T→H exchange was monitored during activation of 210 μM apo-PDC·Mg^{2+} (52 mg mL^{-1}) with 0.5 mM [thiazole-2-T]TDP (1.8 Ci mol^{-1}) at 30 °C in 150 mM sodium MES buffer (pH 6.00), 150 mM pyruvamide, and 15 mM $MgSO_4$. The lines are drawn for first-order rate constants of 0.16 s^{-1} (●) and 0.02 s^{-1} (▽), respectively. Note the difference in scale between the upper and lower panels.

with increasing ionic strength in the range 0.2−2 M (Washabaugh & Jencks, 1989b). The value of k_{obsd} = 1.4 min^{-1} for C(2)-H→T exchange in pyruvamide-activated PDC-bound [thiazole-2-H]TDP agrees with the value of k_{obsd} = 1.6 ± 0.2 min^{-1} for C(2)-H→D exchange in pyruvamide-activated PDC-bound [thiazole-2-H]TDP (Crane et al., 1993). Previous studies showed that there is little or no primary kinetic isotope effect for C(2)-hydron exchange from pyruvamide-activated PDC-bound TDP (Crane et al., 1993). This establishes an effective value of k_{obsd} = 1.7 min^{-1} (=1.4 min^{-1} + 0.32 min^{-1}) for background release of [^{3}H]H_2O produced by parallel enzymic and nonenzymic C(2)-T→H exchange reactions in the absence of substrate (Frost & Pearson, 1961), which corresponds to a half-life of 0.41 min (=0.693/1.7 min^{-1}) under these reaction conditions.

Typical data are shown in Figure 2 (upper panel) for activation of apo-PDC·Mg^{2+} (apo-PDC preincubated with saturating Mg^{2+}) with saturating [thiazole-2-T]TDP in the presence of pyruvamide. Under the reaction conditions of 13:1 TDP/apo-PDC·Mg^{2+}, quantitative activation of apo-PDC·Mg^{2+} with TDP exhibits simple first-order kinetics (k_{obsd} = 0.16 ± 0.01 s^{-1}) in the presence of pyruvamide. This confirms a previous report of quantitative activation of apo-PDC with the cofactors TDP and Mg^{2+} in ≤19 s (k_{obsd} ≥ 0.17 s^{-1}) (Crane et al., 1993) and agrees with a value of k_2 = 0.11 ± 0.06 s^{-1} for the pathway for apo-PDC activation involving Mg^{2+} binding followed by TDP (Vaccaro et al., 1995). This result is important because it establishes a limiting half-life of 4 s (=0.693/0.16 s^{-1}) for activation of apo-PDC in these and other experiments requiring rapid and efficient reconstitution of apo-PDC with TDP and Mg^{2+} in the presence of pyruvamide. The lower panel in Figure 2 shows that nonenzymic and enzymic washout of tritium from [thiazole-2-T]TDP is minimized (6.4%), and activation of apo-PDC·Mg^{2+} with [thiazole-2-T]TDP is maximized (38%), at a time t_1 = 3 s for the activation reaction. Further formation of holo-PDC during the reaction time t_2 (after addition of substrate) was shown to be negligible.

The kinetics of acetaldehyde formation catalyzed by 50 μM pyruvamide-activated PDC (200 μM E active sites) containing [thiazole-2-T]TDP at 30 °C in 100 mM sodium MES buffer (pH 6.00) in H_2O containing 100 mM pyruvamide, 10 mM $MgSO_4$, and 25 μM sodium pyruvate were followed by HPLC under single-turnover conditions ([E] > [S]). Typical data are shown in Figure 3 (lower panel). Under the reaction conditions of 8:1 PDC/pyruvate, the quantitative conversion of pyruvate to form acetaldehyde followed a single exponential (k_{obsd} = 3.8 ± 0.3 s^{-1}) and ≥95% of the acetaldehyde was recovered for HPLC analysis as acetaldehyde (2,4-dinitrophenyl)hydrazone. The experimental value of k_{obsd} = 3.8 ± 0.3 s^{-1} for acetaldehyde formation under these reaction conditions agrees with the value of k_{obsd} = 4.1 s^{-1} calculated using the microscopic rate constants and kinetic mechanism summarized in Scheme 2 for the reaction of 50 μM pyruvamide-activated PDC (*E) with 25 μM pyruvate (S) to form PDC-bound 2-(lact-2-yl)-TDP (*EL), which decarboxylates to form acetaldehyde (P) (Alvarez et al., 1991). The agreement between the experimental and calculated values provides evidence that (1) pyruvamide activates PDC completely without a detectable lag time on the millisecond time scale required for single-turnover experiments (Hübner et al., 1978, 1988; Crane et al., 1993; Harris & Washabaugh, 1995), and (2) apo-PDC·Mg^{2+} was 38% activated with [thiazole-2-T]TDP to form

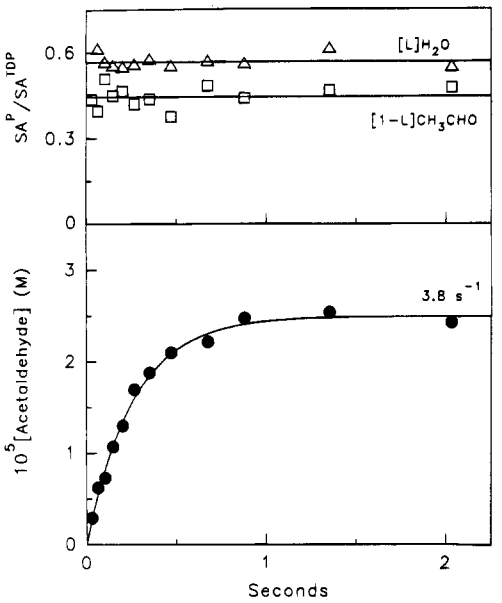


FIGURE 3: Lower panel: formation of [1-L]acetaldehyde (L = H or T) catalyzed by 50 μM pyruvamide-activated holo-PDC (12.0 mg mL^{-1}; 200 μM active sites) containing [thiazole-2-T]TDP (1.8 Ci mol^{-1}) at 30 °C in 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM $MgSO_4$, and 25 μM sodium pyruvate. The line is drawn for a first-order rate constant of 3.8 s^{-1}. Upper panel: dependence of the distribution, SA^P/SA^{TDP}, of tritium transferred from PDC-bound [thiazole-2-T]TDP (SA^{TDP}) to [1-L]-acetaldehyde (□) or [L]H_2O (△) (SA^P) on the extent of the single-turnover reaction with pyruvate (see text). The lines represent the mean values of SA^P/SA^{TDP}.

Scheme 2

$$^*E + S \underset{120\ s^{-1}}{\overset{8.2 \times 10^4\ M^{-1}s^{-1}}{\rightleftharpoons}} {}^*EL \xrightarrow{640\ s^{-1}} {}^*EP \xrightarrow{640\ s^{-1}} {}^*E + P$$

50 μM holo-PDC (see Figure 2). 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 (Crane et al., 1993).

The upper panel in Figure 3 shows the dependence of the distribution, SA^P/SA^{TDP}, of tritium transferred from PDC-bound [thiazole-2-T]TDP (SA^{TDP}) to [1-L]acetaldehyde or [L]H_2O (SA^P) on the extent of the single-turnover reaction with pyruvate (Figure 3, lower panel). The isotopic composition of the [1-L]acetaldehyde product (derived from PDC-bound [C(α)-L]HETDP) and solvent ([L]H_2O) was 43 ± 4% and 54 ± 2%, respectively, of the initial specific radioactivity of the PDC-bound [thiazole-2-T]TDP and was independent of the extent of the reaction. This shows that there is little (≤3%) or no return of the abstracted C(2)-hydron to the C(2) position of PDC-bound TDP.

Nonenzymic C(2)-hydron exchange from free TDP in the quenched samples (pH ≤ 1) is slow ($t_{1/2}$ ≥ 80 days) (Washabaugh & Jencks, 1989a) and T→H exchange involving enolization of pyruvate is slow ($t_{1/2}$ > 8 days) (Alvarez et al., 1991) so these reactions do not interfere with measurements on the seconds time scale. It was previously demonstrated that T→H exchange from electronegative atoms on TDP, substrate, product, and buffer components in the acid-quenched reaction solutions is instantaneous and complete on the seconds time scale (Washabaugh & Jencks, 1989a; Stivers & Washabaugh, 1992; Crane et al., 1993).

DISCUSSION

Catalysis of C(2)-Proton Exchange in TDP. Several groups have evaluated the rate of TDP C(2)-proton exchange and found that the enzyme-mediated reaction of pyruvate is at least $10^{3.5}$ times faster than the maximum rate possible in the nonenzymic reaction. The scale of this rate ratio suggests that a major role of TDP-dependent enzymes might be to change the relative thermodynamic stabilities of TDP and its C(2)-ylide (Washabaugh & Jencks, 1988). An alternate explanation for the relatively fast enzymic proton transfer was offered by several workers, who proposed that the exocyclic 4′-amino group functions as an intramolecular base catalyst for C(2)-proton abstraction in the enzymic (Golbik et al., 1991) and nonenzymic exchange reactions (Petzold et al., 1982; Jordan & Mariam, 1978; Jordan et al., 1982). However, this mechanism is unlikely in aqueous solution because of the low pK_a value of about −0.3 for the protonated 4′-amino group, and the aminopyrimidinyl group has no effect, other than inductive, on the nonenzymic C(2)-proton exchange rate of TDP (Washabaugh & Jencks, 1988) or C(α)-proton exchange rate of HETDP (Stivers & Washabaugh, 1992). Proposed mechanisms to activate the exocyclic 4′-amino group catalytically by attachment of a positive charge at N(1′) by protonation or attachment of the Mg^{2+} ion known to be required for catalysis (Kluger, 1992) or by interaction with an amino acid side chain from the enzyme (Jordan & Mariam, 1978; Ermer et al., 1992; Dyda et al., 1993) are not expected to provide sufficient activation to allow this group to function as an intramolecular general base catalyst for enzymic or nonenzymic C(2)- or C(α)-proton exchange (Washabaugh & Jencks, 1988; Harris & Washabaugh, 1995).

The observed first-order rate constant for C(2)-hydron exchange with solvent in PDC-bound TDP in the absence of substrate, k_{obsd} = 1.4 min^{-1}, is only 4-fold greater than k_{obsd} = 0.32 min^{-1} for catalysis of C(2)-hydron exchange in free TDP in aqueous solution (Figures 1 and 2). The pK_a values of 17.6 and 18.0 for N(1′)-protonated and free TDP indicate that their C(2)-proton transfer reactions are thermodynamically unfavorable in aqueous solution (Washabaugh & Jencks, 1988). The conclusion that protonation of the C(2)-ylide is diffusion-controlled means that proton loss from these carbon acids involves rate-limiting diffusional separation of the proton-transfer products (Washabaugh & Jencks, 1989a). This is important and relevant to the physiological role of TDP because it means that C(2)-proton abstraction occurs at the maximum possible rate for a given equilibrium constant. We conclude that the small amount of observed catalysis of C(2)-hydron exchange from PDC-bound TDP in the absence of substrate 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. However, because the crystallographic structure of PDC in the absence of substrate indicates that the active site and the C(2) position of enzyme-bound TDP are solvent accessible (Dyda et al., 1993), we suggest that the 4-fold rate enhancement of C(2)-hydron exchange in PDC-bound TDP is due to a small (0.6 unit) decrease in the pK_a value for C(2)-H at the active site (Figure 4).

The value of k_{obsd} = 1.4 min^{-1} for C(2)-hydron exchange with solvent in PDC-bound TDP in the absence of substrate is significantly less ($10^{3.5}$-fold) than k_{cat} = 4800 min^{-1} per

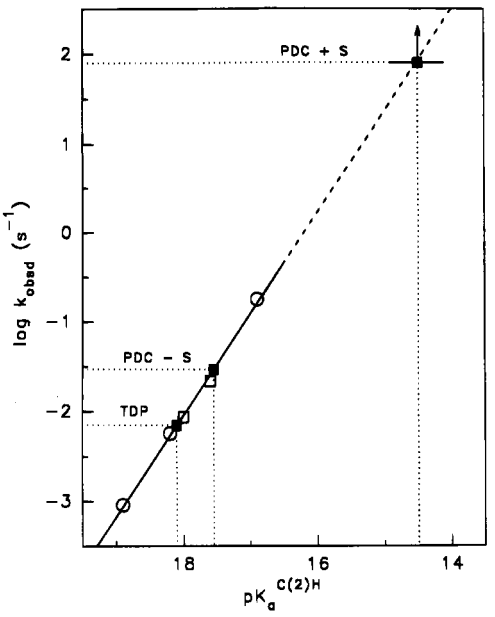


FIGURE 4: Dependence of the rate constants for C(2)-H abstraction from 3-R-4-methylthiazolium ions (○), including N(1′)-unprotonated thiamin (□, $pK_a = 18.0 \pm 0.3$) and N(1′)-protonated thiamin (□, $pK_a = 17.6 \pm 0.3$), on pK_a values for C(2)-H at 30 °C in 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, and 10 mM $MgSO_4$ in H_2O ($I = 0.22$ M); the data are for catalysis by MES and hydroxide ion (Washabaugh & Jencks, 1988, 1989a,b). Values of k_{obsd} for C(2)-hydron abstraction from free N(1′)-unprotonated TDP (■, $pK_a = 18.1$) and PDC-bound TDP in the absence of pyruvate (■, PDC − S) under the above reaction conditions are indicated ($pK_a = 17.5$). The lower limit for catalysis of C(2)-hydron abstraction from PDC-bound TDP in the presence of pyruvate (PDC + S) under the above reaction conditions is indicated by the solid square on the right ($pK_a \leq 14.5$).

active site in tetrameric PDC under these reaction conditions.[4] This provides evidence that C(2)-H in PDC-bound TDP has a pK_a value of ≤14.5 (=18 − 3.5) in the presence of substrate (Figure 4). It is possible that PDC could substantially decrease the pK_a value of enzyme-bound TDP C(2)-H. Spectroscopic and structural studies suggest that the TDP binding site of yeast pyruvate decarboxylase is nonpolar (Wittorf & Gubler, 1970; Dyda et al., 1993). The pK_a decreases markedly (the acid becomes stronger) in environments of low dielectric constant for TDP and other positively charged acids ionizing according to $A^+{-}H + B^- \leftrightarrow A^{\pm} + H{-}B$, where there is a change in the number of ions (Washabaugh & Jencks, 1988).

Aldol-type addition reactions between TDP and carbonyl compounds are catalyzed by several TDP-dependent enzymes, including PDC (Kluger, 1992). It is not known whether the C(2)-ylide exists as a discrete carbanion intermediate on these enzymes. If the C(2)-ylide exists as a discrete intermediate in a stepwise enzymic addition reaction, a pK_a value of ≤14.5 is required for C(2)-H in PDC-bound TDP (Washabaugh & Jencks, 1988). Therefore, either PDC changes the relative thermodynamic stabilities of TDP and the C(2)-ylide or PDC provides a concerted pathway for the addition step that avoids such an unstable carbanion intermediate (Crane & Washabaugh, 1992). Enzymic stabilization of the C(2)-ylide (or destabilization of TDP) in a concerted mechanism could also serve to facilitate proton transfer rather than simply increase the lifetime of the C(2)-ylide for a stepwise mechanism.

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") and details about the relative properties of sequential transition states (Northrop, 1977). There is little or no observed primary kinetic isotope effect, $(k_H/k_D)_{obsd} \leq 1.2$ (Crane et al., 1993), for C(2)-L abstraction from [thiazole-2-L]TDP during a single turnover or hydron transfer to the C(2) position of PDC-bound TDP in isotopically labeled solvent, $(k_H/k_T)_{obsd} = 1.0$, under single- or multiple-turnover conditions (Harris & Washabaugh, 1995). Incomplete C(2)-hydron exchange from PDC-bound TDP under the conditions of these experiments (Figure 2) provides evidence against facile C(2)-L→H exchange at the enzyme active site significantly depressing the actual value of $(k_H/k_L)_{obsd}$ because of washout of the isotopic label before turnover. Other possible reasons for the small isotope effect have been discussed in detail (Crane et al., 1993).

The absence of a primary kinetic isotope effect for C(2)-hydron transfer to and from PDC-bound TDP is consistent with a concerted enzymic addition mechanism, a stepwise enzymic addition mechanism involving rapid equilibrium formation of the C(2)-ylide as a discrete intermediate followed by rate-limiting carbon−carbon bond formation, or a rate-limiting conformational change preceding addition of TDP to the substrate carbonyl group (Crane et al., 1993). The observed isotope effect is expected to approach the equilibrium isotope effect for C(2)-hydron abstraction if PDC uses a stepwise mechanism involving rapid equilibrium formation of the C(2)-ylide before carbon−carbon bond formation. The fractionation factor is 0.98 ± 0.06 for C(2)-H→D exchange from TDP in aqueous solution (Harris & Washabaugh, 1995).

The C(2)-ylide is not the "resting state" of TDP bound to pyruvamide-activated PDC because the abstracted C(2)-hydron is transferred, in part, to the C(α) position of enzyme-bound HETDP in the course of a single turnover (see below) or multiple enzymic turnovers (Harris & Washabaugh, 1995). These results require solvent-derived hydron transfer to the C(2) position of PDC-bound TDP to regenerate the coenzyme in the catalytic cycle and provide evidence against enzymic mechanisms involving a stable PDC-bound C(2)-ylide before substrate binding at the active site.

Fate of the C(2)-Proton. The C(2)-hydron abstracted from PDC-bound [thiazole-2-L]TDP has three possible fates during acetaldehyde formation catalyzed by PDC under single-turnover conditions. First, the abstracted C(2)-hydron may be quantitatively transferred to the C(α) position of PDC-bound HETDP, either directly from the conjugate acid of the catalytic base or through the mediation of another catalytic group, to ultimately form [1-L]acetaldehyde (Scheme 1). Second, the conjugate acid of the catalytic base may completely or partially exchange the label for a solvent-derived proton from another catalytic group of the enzyme or solvent, ultimately forming unlabeled or partially labeled [1-L]acetaldehyde. Third, the abstracted C(2)-hydron may be quantitatively returned to the C(2) position of PDC-bound TDP upon carbon−carbon bond cleavage in PDC-bound HETDP to form [1-H]acetaldehyde. In this case hydron transfer to the C(α) position of PDC-bound HETDP would involve only a solvent-derived proton. The extent of label

[4] The value of $k_{cat} = 4800$ min^{-1} was calculated by assuming a maximal specific activity for pure holo-PDC of 320 U mg^{-1} (Sieber et al., 1983), four active sites per tetramer, and a molecular mass of 242 kDa for the holoenzyme (Ullrich & Freisler, 1977; Hopmann, 1980; Sieber et al., 1983).

transfer from the C(2) position of PDC-bound [thiazole-2-L]TDP to the C(α) position of HETDP, and subsequently [1-L]acetaldehyde, will reflect the competition between the three possible routes for the C(2)-hydron in a single PDC turnover.

Figure 3 shows that the isotopic label was transferred from the C(2) position of PDC-bound [thiazole-2-T]TDP to either [1-L]acetaldehyde (43 ± 4%) or solvent (54 ± 2%) with little (≤3%), if any, return of the abstracted C(2)-hydron to the C(2) position of PDC-bound TDP after a single turnover. The *partial* exchange of C(2)-derived tritium into solvent requires that hydron transfer from C(2) occurs to a catalytic base in which the conjugate catalytic acid is (partially) shielded from hydron exchange with *bulk* solvent. If isotopic exchange occurred between the conjugate catalytic acid and bulk solvent, then all of the tritium label would be "washed out" into solvent and no label would have appeared in the acetaldehyde product in a single turnover. Incomplete C(2)-hydron exchange from PDC-bound TDP under the conditions of these experiments provides evidence against facile C(2)-T→H exchange at the enzyme active site significantly affecting the distribution of tritium derived from enzyme-bound [thiazole-2-T]TDP because of washout of the isotopic label before turnover.

The partial transfer (43%) of the abstracted C(2)-hydron to HETDP must involve competition between the abstracted C(2)-hydron and another unlabeled (solvent-derived) proton. There are several possible mechanisms to account for partial exchange of the abstracted C(2)-hydron for solvent-derived proton(s): (1) a diprotic group containing both the abstracted C(2)-hydron and a hydron equilibrated with solvent, which would have a 1:2 probability of transfer of the label originating from either the C(2) position of TDP or solvent; (2) C(α)-hydron transfer from either the conjugate acid of the monoprotic group that abstracted the C(2)-hydron or a second monoprotic acid catalyst equilibrated with solvent; or (3) C(α)-hydron transfer occurring from the conjugate acid of the monoprotic group that abstracted the C(2)-hydron and undergoing exchange with a solvent molecule in the active site or a second monoprotic group that is equilibrated with, but shielded from, bulk solvent. In this case either group could act as the single acid catalyst for hydron transfer at the C(α) position of HETDP. If either of the catalytic groups containing the abstracted C(2)-hydron has a fractionation factor different than unity, accumulation of <50% of the label would be observed in the [1-L]acetaldehyde product. Minimally, these results require that hydron transfer to the C(2) position to regenerate the coenzyme for the next catalytic turnover must occur from a catalytic group (either from solvent directly or from a second catalytic acid of the enzyme that undergoes rapid hydron exchange with the solvent) separate and distinct from the catalytic group involved in C(2)-hydron abstraction and addition to the reactive carbonyl group of pyruvate. We conclude that the abstracted C(2)-hydron is involved in the specific protonation of the C(α) position of the PDC-bound intermediate HETDP, which is cleaved to form [1-L]acetaldehyde and PDC-bound [thiazole-2-H]TDP. The source of catalytic protons for protonation of the C(2) position in TDP and the C(α) position in HETDP will be examined further in the following paper.

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