Solvent-Derived Protons in Catalysis by Brewers' Yeast Pyruvate Decarboxylase†

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ABSTRACT: Catalysis of proton transfer to thiamin diphosphate (TDP) and 2-(1-hydroxyethyl)thiamin diphosphate (HETDP) by pyruvate decarboxylase isozymes (PDC; EC 4.1.1.1) from *Saccharomyces carlsbergensis* was investigated by determining the solvent discrimination tritium isotope effect, $(k_H/k_T)_{disc}$, on the reaction of pyruvate to form acetaldehyde in the presence of the nonsubstrate allosteric effector pyruvamide. The fractionation factors for TDP C(2)-L ($\phi_{C(2)} = 0.98 \pm 0.06$) and HETDP C(α)-L ($\phi_{C(\alpha)} = 1.01 \pm 0.07$) (L = H or D) do not contribute significantly to observed enzymic isotopic discrimination. The value of $(k_H/k_T)_{disc} = 1.0$ for reprotonation of TDP C(2)-L under single-turnover conditions ([E] > [S]) is consistent with C(2)-hydron transfer via a catalytic group ($\phi = 1$) equilibrated with solvent. [1-L]Acetaldehyde formation under transient steady-state ([E] < [S]) conditions shows solvent discrimination tritium isotope effects that increase over the range $(k_H/k_T)_{disc} = 0.39$ (single turnover) to 0.86 (ten turnovers). The 2-fold increase in the value of $(k_H/k_T)_{disc}$ for the [1-L]acetaldehyde product under steady-state compared to single-turnover conditions is attributed to a fractionation factor of $\phi_1 = 0.88 \pm 0.06$ for the residue(s) involved in C(α)-hydron transfer to form HETDP. This provides evidence that catalysis of acetaldehyde formation by PDC involves specific protonation of both HETDP C(α)-L and TDP C(2)-L ($\phi_2 = 1.0 \pm 0.1$) and requires at least two catalytic groups. Values of $\phi \leq 1$ for protonation of TDP C(2)-L and HETDP C(α)-L provide no evidence that the exocyclic 4′-amino or -imino group ($\phi \geq 1.2$) provides significant intramolecular catalysis in the enzyme-bound coenzyme.

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 as follows: $CH_3C(O)CO_2^- + H^+ \rightarrow CH_3CHO + CO_2$ (Alvarez et al., 1991, 1995; Crane et al., 1993) (Scheme 1). The preceding paper describes evidence that (1) hydron[2] (L = H or T) transfer from the C(2) position of PDC-bound [thiazole-2-L]TDP occurs to a catalytic base in which the conjugate catalytic acid is shielded from hydron exchange with bulk solvent, (2) the conjugate catalytic acid is involved in transfer of the C(2)-derived hydron to the C(α) position of the PDC-bound intermediate 2-(1-hydroxyethyl)thiamin diphosphate (HETDP, **2a**), and (3) hydron transfer occurs to the C(2) position of PDC-bound TDP to regenerate the coenzyme for the following turnover. The goal of the work reported here was to monitor discrimination between solvent-derived hydrons in the formation of PDC-bound [thiazole-2-L]TDP and [1-L]acetaldehyde from pyruvate catalyzed by PDC in isotopically labeled solvent and further 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 show that the fractionation factors[3] for the C(2) position in TDP and the C(α) position in HETDP do not contribute to observed enzymic isotopic discrimination in the reaction products. Discrimination between hydrons in the formation of [thiazole-2-L]TDP and [1-L]acetaldehyde from pyruvate catalyzed by PDC in isotopically labeled solvent can result from (1) expression of the primary kinetic isotope effect for hydron transfer to that product or (2) a functional group with a fractionation factor not equal to unity (Albery & Knowles, 1976; Fletcher et al., 1976; Maister et al., 1976; Yamada & O'Leary, 1977; Northrop, 1981). The magnitudes of isotopic discrimination in the PDC-catalyzed reaction of TDP with pyruvate under single-turnover and transient steady-state conditions in $[^3H]H_2O$ provide (1)

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[1] Abbreviations: PDC, pyruvate decarboxylase; TDP, thiamin diphosphate; HETDP, 2-(1-hydroxyethyl)thiamin diphosphate; HET, 2-(1-hydroxyethyl)thiamin; oxyHET, 2-(1-hydroxyethyl)oxythiamin; MES, 2-(*N*-morpholino)ethanesulfonate; Me_2SO, dimethyl sulfoxide; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; ADH, alcohol dehydrogenase; NADH (NAD^+), reduced (oxidized) nicotinamide adenine dinucleotide; DMT, 3,4-dimethyl-5-(2-pyrophosphoethyl)thiazolium ion; AP, 4-amino-2-methylpyrimidine.

[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.

[3] The fractionation factor, ϕ, is the equilibrium isotope effect for exchange of a hydron by a functional group (S−L) with a solvent molecule (ROL):

$$\text{S-H + ROD} \overset{\phi}{\rightleftharpoons} \text{S-D + ROH}$$

ϕ quantifies the preference of the hydron site in the functional group for a deuteron (or triton) over a proton relative to that for the hydron site in the solvent (eq 1) [see, for example, Schowen and Schowen (1982)].

$$\phi = \frac{[S-D][ROH]}{[S-H][ROD]} \quad (1)$$

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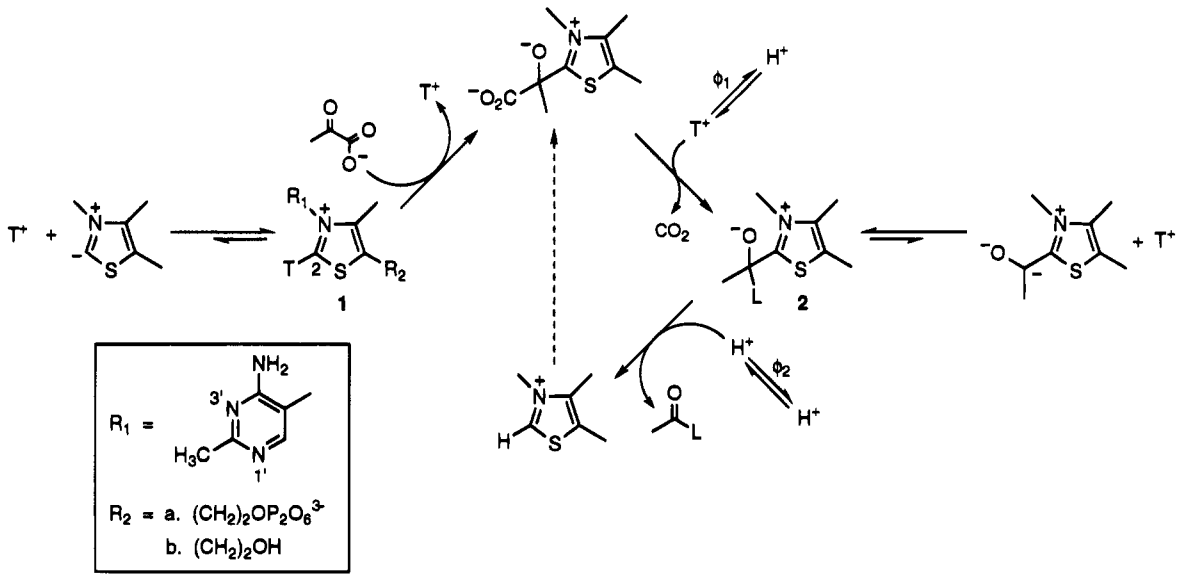
Scheme 1



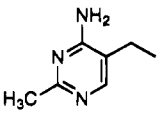
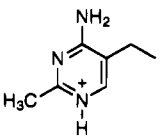
evidence for a fractionation factor of $\phi_1 = 0.88$ for the residue(s) involved in C(α)-hydron transfer to form enzyme-bound HETDP, (2) evidence for at least two catalytic groups in catalysis of acetaldehyde formation and specific protonation of HETDP C(α)-L and TDP C(2)-L ($\phi_2 = 1.0$), and (3) no evidence that the aminopyrimidinyl group contributes significant intramolecular catalysis for hydron transfer to and from enzyme-bound TDP C(2)-L or HETDP C(α)-L.

EXPERIMENTAL PROCEDURES

Materials and experimental procedures were generally as described in the preceding paper (Harris & Washabaugh, 1995). All operations with volatile radioactivity were performed in a hood. Dimethyl-d_6 sulfoxide (99.5+ atom % D) (Me_2SO-d_6), acetic-d_3 acid-d (99.5 atom % D), deuterium oxide (99.9 atom % D), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) were purchased from Aldrich. [^{3}H]H_2O (100 mCi g^{-1}) was purchased from New England Nuclear. Thiamin chloride hydrochloride (**3b**) (see Table 1) [**1b**, N(1′)-unprotonated (pK_a = 5.1) (Washabaugh & Jencks, 1988)] was recrystallized from methanol/ethanol: mp 242−243 °C. The synthesis of 3,4-dimethylthiazolium chloride (**4**) (Crosby et al., 1970) and 3-(cyanomethyl)-4-methylthiazolium chloride (**5**) (Washabaugh & Jencks, 1988) has been described. The synthesis of 2-(1-hydroxyethyl)oxythiamin chloride hydrochloride (oxyHET) (**6**), 2-(1-hydroxyethyl)-3-(4-nitrobenzyl)-4-methylthiazolium chloride (**7**), and 2-(1-hydroxyethyl)-3-(pentafluorobenzyl)-4-methylthiazolium bromide (**8**) has also been described (Stivers & Washabaugh, 1992) (see Table 2). Brewers' yeast holo-PDC (45−60 units mg^{-1}) was prepared as described previously (Sieber et al., 1983). "Unresolved" PDC containing the α_4 and $\alpha'_2\beta_2$ isozymes was used in the experiments reported here (Kuo et al., 1986).

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). The value of pL (L = H or D) was obtained by adding $(\Delta pH)_n = 0.076n^2 + 0.3314n$ to the observed pH of mixed H_2O−D_2O solutions (n = 0.20−0.98 atom fraction D) (Schowen & Schowen, 1982). The preparation of mixed H_2O−D_2O solutions and determination of the deuterium content of isotopic water have been described (Schowen & Schowen, 1982).

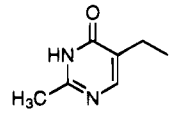
Table 1: Fractionation Factors for Nonenzymic C(2)-H→D Exchange in 3-R-4-methylthiazolium Ions[a]

	R	p$K_a^{C(2)H}$[b]	$\phi_{C(2)}$
5	CH_2CN	16.9[c]	0.994 ± 0.018[c]
1b		18.0[d]	0.983 ± 0.022[d]
		17.7[e]	0.995 ± 0.008[e]
		17.7[f]	0.955 ± 0.018[f]
3b		17.6[c]	0.971 ± 0.014[c]
4	Me	18.9[c]	1.03 ± 0.04[c]

[a] The fractionation factor, ϕ, is defined in footnote 3 and eq 1 (L = H or D); values of $\phi_{C(2)}$ are expressed as the mean ± standard error of the mean (N = 5). Experiments were performed in mixed H_2O−D_2O solutions (0.20−0.80 atom fraction D) at the indicated apparent pH (pL) (see text). [b] Washabaugh & Jencks, 1988, 1989b. We estimate that these pK_a values are accurate to within ±0.3. [c] 100 mM potassium acetate-d_3 buffer (pL 3.8) at 30 °C, I = 1.0 M (KCl). [d] 100 mM potassium phosphate buffer (pL 6.40) at 30 °C, I = 1.0 M (KCl). [e] 100 mM potassium phosphate buffer (pL 6.90) at 30 °C, I = 200 mM (KCl). [f] 100 mM potassium phosphate buffer (pL 6.90) containing 1.0 M Me_2SO-d_6 at 30 °C, I = 200 mM (KCl).

Determination of Fractionation Factors. Fractionation factors[3] for C(2)-H→D exchange in 3-R-4-methylthiazolium ions and C(α)-H→D exchange in 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions were determined by integrating the corresponding ^{1}H-NMR peaks at equilibrium in mixed H_2O−D_2O solutions (Schowen & Schowen, 1982) at 30 °C in the presence and absence of 1.0 M Me_2SO-d_6. The ionic strength was maintained at 200 mM or 1.0 M with KCl. The exchange reaction was initiated by dissolving 0.10 mmol of

Table 2: Fractionation Factors for Nonenzymic C(α)-H→D Exchange in 2-(1-Hydroxyethyl)-3-R-4-methylthiazolium Ions[a]

	R	$pK_a^{C(\alpha)H}$[b]	$\phi_{C(\alpha)}$
8	$C_6F_5CH_2$	18.7	1.01 ± 0.04
7	4-NO_2-Bzl	19.3	0.98 ± 0.04
6		19.8	1.03 ± 0.04
		19.5[c]	1.03 ± 0.04[c]
		19.5[d]	1.03 ± 0.04[d]

[a] The fractionation factor, ϕ, is defined in footnote 3 and eq 1 (L = H or D); values of $\phi_{C(\alpha)}$ are expressed as the mean ± standard error of the mean ($N = 5$). Experiments were performed in mixed H_2O-D_2O solutions (0.23−0.98 atom fraction D) containing 100 mM potassium phosphate buffer (pL 6.90) at 30 °C, $I = 1.0$ M (KCl) unless otherwise stated (see text). [b] Stivers & Washabaugh, 1992. We estimate that these pK_a values are accurate to within ±0.2. [c] 100 mM potassium phosphate buffer (pL 6.90) at 30 °C, $I = 200$ mM (KCl). [d] 100 mM potassium phosphate buffer (pL 6.90) containing 1.0 M Me_2SO-d_6 at 30 ° C, $I =$ 200 mM (KCl).

thiazolium salt in 1 mL of buffer (containing weighed fractions of H_2O- and D_2O-containing buffers of the same composition), giving a final concentration of 100 mM thiazolium salt. The NMR tube was placed in a constant temperature bath (30 ± 0.2 °C), and the tube was removed for about 30 min every 1−3 days in order to measure the integrated areas of the signals. Integrated areas were measured for the C(2)-H signal (δ = 9.3−10.3 ppm) (**1b**, **3b**, **4**, **5**) or C(α)-H signal (δ = 5.4−5.7 ppm) (**6**−**8**) and compared to those for C(6′)-H of the aminopyrimidinyl moiety (δ = 7.7−8.1 ppm) (**1b**, **3b**, **6**), C(4)-methyl protons (δ = 2.2−2.8 ppm) (**4**, **5**), or C(5)-proton (δ = 7.7−7.9 ppm) (**7**, **8**) (as nonexchanging internal standards).

Determination of Solvent Discrimination Isotope Effects. The solvent discrimination tritium isotope effect, $(k_H/k_T)_{disc}$, on C(2)-H→T transfer to TDP and C(α)-H→T transfer to HETDP in rapid-quench kinetic experiments was determined from the ratio of the specific radioactivities of the [thiazole-2-T]TDP or [1-T]acetaldehyde product and the solvent. "Reaction buffer" refers to 100 mM sodium MES buffer (pH 6.00) in H_2O containing 100 mM pyruvamide and 10 mM $MgSO_4$. 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 45 μL of 1.25 or 50 μM holo-PDC in 200 mM sodium MES buffer (pH 6.00) containing 200 mM pyruvamide, 0 or 20 mM TDP, and 20 mM $MgSO_4$. The other sample loop contained 45 μL of 50 μM sodium pyruvate in $[^3H]H_2O$ (1.7 Ci mol^{-1}). The decarboxylation reaction was initiated by mixing the contents of the sample loops that was allowed to react for a time t ($0.06 < t < 4$ s for single-turnover experiments; $1 < t < 90$ s for transient steady-state experiments) before the reaction was acid-quenched. For PDC-catalyzed acetaldehyde formation under single-turnover conditions ([E active sites] > [pyruvate]), the final concentrations were 25 μM holo-PDC (100 μM E active sites), 100 mM sodium MES buffer (pH 6.00), 25 μM sodium pyruvate, 100 mM pyruvamide, and 10 mM $MgSO_4$ in $[^3H]H_2O$ (0.85 Ci mol^{-1}). For PDC-catalyzed acetaldehyde formation under transient steady-state conditions ([E active sites] < [pyruvate]), the final concentrations were 0.625 μM holo-PDC (2.5 μM E active sites), 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM TDP, 10 mM $MgSO_4$, and 25 μM sodium pyruvate in $[^3H]H_2O$ (0.85 Ci mol^{-1}). Control reactions for all experiments were performed in the absence of pyruvate to determine background radioactivity, if any, incorporated at C(2) or C(α). 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 ≥20 min at 16000g. Values of $(k_H/k_T)_{disc}$ for C(2)-H→T transfer to PDC-bound TDP were calculated with $(k_H/k_T)_{disc} = (SA^{TDP}/SA^S)$, in which SA^{TDP} is the specific radioactivity of the [thiazole-2-T]TDP (see below) and SA^S is the initial specific radioactivity of the solvent. Values of $(k_H/k_T)_{disc}$ for C(α)-H→T transfer to PDC-bound HETDP were calculated with $(k_H/k_T)_{disc} = (SA^A/SA^S)$, in which SA^A is the specific radioactivity of the [1-T]acetaldehyde product (see below).

Determination of Acetaldehyde. [1-L]Acetaldehyde (L = H or D) was determined with MBTH (Paz et al., 1965) or yeast alcohol dehydrogenase (ADH; alcohol:NAD^+ oxidoreductase; EC 1.1.1.1) and NADH (Ullrich, 1970). [1-T]-Acetaldehyde produced at each time point t (≤2.25 nmol) was determined as the (2,4-dinitrophenyl)hydrazone by HPLC after an extraction step as described previously (Crane et al., 1993) except that a 25-μL injection of the "spiked" and extracted sample was analyzed by reversed-phase (C_{18}) HPLC on a Whatman column (4.6 × 250 mm) with isocratic (45:55 water/acetonitrile) elution at ambient temperature and detection at 360 nm (0.010 AUFS); ≥95% of the acetaldehyde was recovered for HPLC analysis. [1-T]Acetaldehyde (2,4-dinitrophenyl)hydrazone specific radioactivity was determined by mixing a 150-μL aliquot of the spiked and extracted sample with 10 mL of EcoLite(+) scintillation cocktail (ICN Biomedicals), counting the sample for at least 10^4 counts with automatic quench control, and dividing the total radioactivity recovered in [1-T]acetaldehyde (2,4-dinitrophenyl)hydrazone-containing fractions (after subtraction of background counts) by the amount of acetaldehyde formed at the corresponding time point. A 150-μL aliquot of the spiked and extracted sample typically gave ≤2000 DPM. No radioactivity was detected in the spiked and extracted samples derived from control reactions in the absence of pyruvate. All radioactivity in the spiked and extracted samples derived from the pyruvate reaction was contained in the [1-T]acetaldehyde (2,4-dinitrophenyl)-hydrazone-containing fractions.

Determination of TDP. [Thiazole-2-T]TDP in the quenched samples was determined by measuring nonvolatile, exchanged tritium derived from PDC-bound TDP. A 150-μL aliquot of the quenched sample for each time point was mixed with 150 μL of 1 M HCl, and the solvent (which contained $[^3H]H_2O$) was removed by evaporation in a Savant Speed-Vac centrifugal concentrator; this process was repeated until a 150-μL aliquot of the sublimate contained ≤12 000 DPM of volatile radioactivity. [Thiazole-2-T]TDP was isolated by dissolving the nonvolatile residue in 150 μL of 1 M HCl and spin column cation-exchange chromatography on Dowex AG 50W-X4 (H^+ form) (Washabaugh & Collins, 1986). The spin column was washed with 150 μL of 1 M HCl until the eluate contained no significant radioactivity (shown to be associated with buffer components), and

[thiazole-2-T]TDP was eluted with 150 μL of 2 M $MgCl_2$ containing 1 M HCl. A 600-μL aliquot of the TDP-containing eluate (≥95% recovery of PDC-bound TDP) was counted for at least 10^4 counts with automatic quench control. [Thiazole-2-T]TDP specific radioactivity 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 acetaldehyde formed at the corresponding time point. A 600-μL aliquot of the TDP-containing eluate typically gave ≤2000 DPM after subtraction of background counts. Background radioactivity (≤400 DPM) in the TDP-containing eluates was determined in control reactions performed in the absence of pyruvate. All radioactivity in the TDP-containing eluates was shown to be contained in TDP-containing fractions by reversed-phase (C_{18}) HPLC (Kimura & Itokawa, 1983). Parallel experiments in deuterium oxide and ^{1}H-NMR examination of the exchanged TDP showed that isotope labeling under these conditions occurs exclusively by exchange at the C(2) position (Crane et al., 1993).

Data Analysis. Quantitative conversion of pyruvate to acetaldehyde under single-turnover and transient steady-state conditions followed a single exponential, k_{obsd}, with an offset for plots of [acetaldehyde] against time (≥13 time points). The offset was shown to represent background [acetaldehyde]. Where multiple determinations of initial rates and values of k_{obsd} were made, they agreed within ±10% of the mean value. Values are reported as the mean ± standard error of the mean.

RESULTS

Fractionation factors, $\phi_{C(2)}$, for nonenzymic C(2)-H→D exchange in several 3-R-4-methylthiazolium ions (**1b**, **3b**, **4**, **5**), including N(1′)-protonated thiamin (**3b**; see Table 1) and N(1′)-unprotonated ("free") thiamin (**1b**), at equilibrium in mixed H_2O-D_2O solutions at 30 °C and ionic strength 0.2−1.0 M, maintained with potassium chloride, in the presence and absence of 1.0 M Me_2SO-d_6 were determined by ^{1}H-NMR spectroscopy. Typical data are shown in Figure 1 (lower panel) for C(2)-H→D exchange in **1b**. Experiments with other 3-R-4-methylthiazolium ions were carried out in the same manner as those shown for **1b** with the same number of points, and C(2)-H→D exchange achieved equilibrium in ≤2 days. Values of $\phi_{C(2)}$ are summarized in Table 1. The value of $\phi_{C(2)} = 0.971 \pm 0.014$ for **3b** agrees with a previous estimate of $\phi_{C(2)} = 0.97 \pm 0.05$ (Alvarez et al., 1991). There is no significant change in $\phi_{C(2)}$ (= 0.98 ± 0.06) with increasing ionic strength (0.2−1.0 M), increasing pK_a of the C(2) position (16.9−18.9), increasing organic solvent (0−1.0 M) in the reaction medium, or a change in the 5-substituent (H to CH_2CH_2OH) in the thiazolium ring (Table 1 and Figure 1; upper panel). It was shown previously that the kinetics of C(2)-H→D exchange is independent of a change in the 5-substituent (H to $CH_2CH_2OP_2O_6^{3-}$) in the thiazolium ring (Washabaugh & Jencks, 1988). This shows that medium effects on $\phi_{C(2)}$ are small and that $\phi_{C(2)} = 0.98 \pm 0.06$ for nonenzymic C(2)-H→D exchange in N(1′)-protonated (**3a**) or free TDP (**1a**).

Fractionation factors, $\phi_{C(\alpha)}$, for nonenzymic C(α)-H→D exchange in several 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions (**6**−**8**) at equilibrium in mixed H_2O-D_2O solutions at 30 °C and ionic strength 0.2−1.0 M, maintained with potassium chloride, in the presence and absence of 1.0 M Me_2SO-d_6 were determined by ^{1}H-NMR spectroscopy. Typical data are shown in Figure 2A for C(α)-H→D exchange in oxyHET (**6**) and parallel cleavage of oxyHET to acetaldehyde and oxythiamin in 100 mM potassium phosphate buffer (pD 6.90) in D_2O at 30 °C, I = 1.0 M (KCl). The formation of [1-L]acetaldehyde (L = H or D) and disappearance of [C(α)-H]oxyHET were monitored, and the fraction of [C(α)-D]oxyHET in a series first-order reaction (Frost & Pearson, 1961) (Scheme 2) was calculated using the fraction of [C(α)-H]oxyHET and acetaldehyde determined at each time point. The curves for the reactions of oxyHET were calculated by numerical integration using the independently determined rate constants for C(α)-H→D exchange, $k_1 = 5.8 \times 10^{-7}$ s^{-1} (Stivers & Washabaugh, 1992), and cleavage, $k_2 = 6.0 \times 10^{-8}$ s^{-1} (Crane & Washabaugh, 1992), summarized in Scheme 2. Experiments with other 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions were carried out in the same manner as those shown for **6** (Figure 2) with the same number of points, and C(α)-H→D exchange achieved equilibrium in ≤150 days ($k_1 \geq 10k_2$). Values of $\phi_{C(\alpha)}$ are summarized in Table 2. The value of $\phi_{C(\alpha)} = 1.03 \pm 0.04$ for **6** (Figure 2B, lower panel) is in reasonable agreement with a calculated value of $\phi \approx 1.1$.[4]

There is no significant change in $\phi_{C(\alpha)}$ (=1.01 ± 0.07) with increasing ionic strength (0.2−1.0 M), increasing pK_a of the C(α) position (18.7−19.8), increasing organic solvent (0−1.0 M) in the reaction medium, or a change in the 5-substituent (H or CH_2CH_2OH) in the thiazolium ring (Table 2 and Figure 2B, upper panel). It was shown previously that the kinetics of C(α)-proton transfer is independent of a

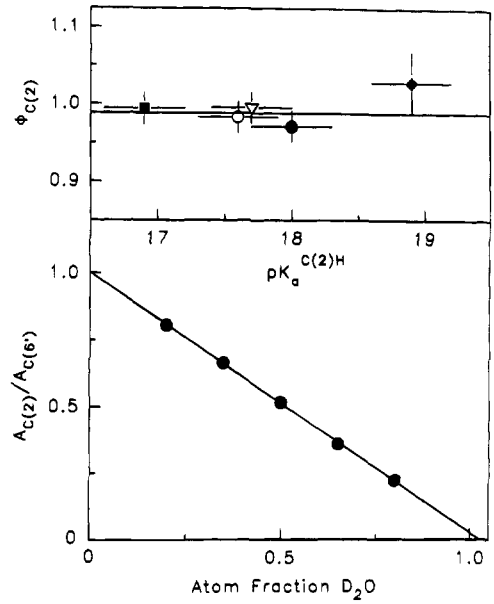


FIGURE 1: Lower panel: dependence of $A_{C(2)}/A_{C(6')}$ at equilibrium on the atom fraction D_2O, where A is the integrated area of the C(2)-H or C(6′)-H signal, respectively, of thiamin in 100 mM potassium phosphate buffer (pL 6.40) at 30 °C, I = 1.0 M (KCl). The slope of the line gives the fractionation factor for nonenzymic C(2)-H→D exchange in N(1′)-unprotonated ("free") thiamin, $\phi_{C(2)}$, which is defined in eq 1 (see text). Upper panel: dependence on the p$K_a^{C(2)H}$ value of nonenzymic $\phi_{C(2)}$ values for 3-R-4-methylthiazolium ions: N(1′)-protonated thiamin (**3b**) (○); free thiamin (I = 1.0 M) (**1b**) (●); free thiamin (I = 200 mM) (▽); R = CH_2CN (**5**) (■); and R = Me (**4**) (◆). Values of $\phi_{C(2)}$ are summarized in Table 1.

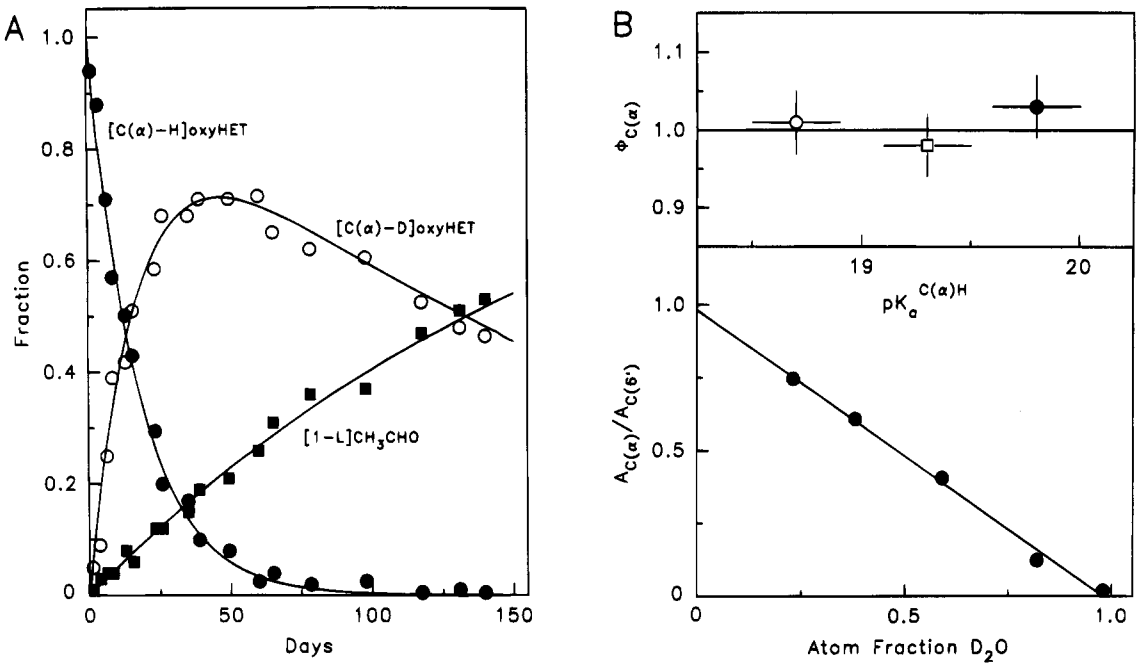
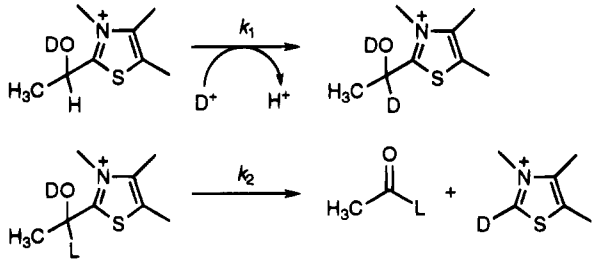


FIGURE 2: (A) C(α)-H→D exchange in 100 mM 2-(1-hydroxyethyl)oxythiamin (oxyHET) (**6**) and cleavage of oxyHET to acetaldehyde and oxythiamin in 100 mM potassium phosphate buffer (pD 6.90) in D_2O at 30 °C, I = 1.0 M (KCl). The formation of acetaldehyde (■) and disappearance of [C(α)-H]oxyHET (●) were monitored. The fraction of [C(α)-D]oxyHET (○) in a series first-order reactions (Scheme 2) was calculated using the fraction of [C(α)-H]oxyHET and acetaldehyde determined at each time point (see text). The curves were calculated by numerical integration using the rate constants summarized in Scheme 2. (B) Lower panel: dependence of $A_{C(\alpha)}/A_{C(6')}$ at equilibrium on the atom fraction D_2O, where A is the integrated area of the C(α)-H or C(6′)-H signal, respectively, of oxyHET in 100 mM potassium phosphate buffer (pL 6.90) at 30 °C, I = 1.0 M (KCl). The slope of the line gives the fractionation factor for nonenzymic C(α)-H→D exchange in N(1′)-unprotonated ("free") oxyHET, $\phi_{C(\alpha)}$, which is defined in eq 1 (see text). Upper panel: dependence on the $pK_a^{C(\alpha)H}$ value of nonenzymic $\phi_{C(\alpha)}$ values for 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions: R = $C_6F_5CH_2$ (**8**) (○), R = 4-NO_2-Bzl (**7**) (□), and free oxyHET (**6**) (●). Values of $\phi_{C(\alpha)}$ are summarized in Table 2.

Scheme 2



change in the 5-substituent (H to $CH_2CH_2OP_2O_6^{3-}$) in the thiazolium ring (Stivers & Washabaugh, 1992). We conclude that medium effects on $\phi_{C(\alpha)}$ are small and that $\phi_{C(\alpha)}$ = 1.01 ± 0.07 for nonenzymic C(α)-H→D exchange in N(1′)-protonated or free HETDP (**2a**).

C(α)-H→D exchange from free oxyHET (**6**) was used as a model for free HET (**2b**) because of a rapid side reaction involving the exocyclic 4′-amino group (Stivers & Washabaugh, 1992): the side reaction was not observed for **6** (Figure 2A). The structurally similar free 3-aminopyrimidinyl and 3-oxopyrimidinyl substituents (see Tables 1 and 2) in **2b** and **6**, respectively, have identical steric and electronic effects on rates of C(α)-proton transfer from **2b** and **6**: (1) the 3-substituents of **2b** and **6** have identical values of the inductive substituent constant σ_I (Washabaugh et al., 1993); (2) **2b** and **6** have identical second-order rate constants for catalysis by deuteroxide ion of C(α)-proton transfer in D_2O (Stivers & Washabaugh, 1992); and (3) the rate constants for catalysis of C(α)-proton transfer from N(1′)-protonated HET and **6** by hydroxide ion or phosphate dianion follow the same Hammett correlation with σ_I for the 3-substituent (Stivers & Washabaugh, 1992). N(1′)-protonated HET ($pK_a^{C(\alpha)H}$ = 18.4) was not studied here because C(α)-H→D exchange in this substrate was previously shown to be unacceptably slow ($t_{1/2} \geq$ 270 days) (Stivers & Washabaugh, 1992).

The kinetics of [1-L]acetaldehyde (L = H or T) formation catalyzed by pyruvamide-activated PDC containing [thiazole-2-H]TDP at 30 °C in [^{3}H]H_2O containing 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM $MgSO_4$, and 25 μM sodium pyruvate were followed by HPLC under single-turnover conditions. Typical data are shown in Figure 3 (lower panel) for decarboxylation of 25 μM sodium pyruvate to form acetaldehyde catalyzed by 25 μM pyruvamide-activated PDC (100 μM active sites). Under the reaction conditions of 4:1 PDC/pyruvate, the quantitative conversion of pyruvate to form acetaldehyde followed a single exponential (k_{obsd} = 1.7 ± 0.2 s^{-1}), and ≥95% of the

[4] The calculated value of $\phi \approx 1.1$ [=(0.80)(1.18)(1.10)(1.09)] for C(α)-H→D exchange in free HET was estimated using $\phi = \phi_{CH4}\prod s_i$ with ϕ_{CH4} = 0.80 and substituent effects (s_i) on C−H bond fractionation factors of s = 1.18 for −OH and s = 1.10 for $-CH_3$ as suggested for O and C substituents, respectively (Kresge et al., 1987). The value of s = 1.04 for a −CN group and the suggested value of s = 1.13 for a N(+) substituent were used to estimate the value of s = 1.09 [=(1.04 + 1.13)/2] for the thiazolium ring (Kresge et al., 1987). The activating effect for proton transfer of the thiazolium ring is significantly larger than that of a −CN group (Washabaugh & Jencks, 1989), and the largest factor contributing to fast proton transfer from 2-acetyl-3-R-thiazolium ions (Halkides et al., 1993) and 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions, including HET (Stivers & Washabaugh, 1991, 1992), is the through-space electrostatic effect of the positive charge in the thiazolium ring.

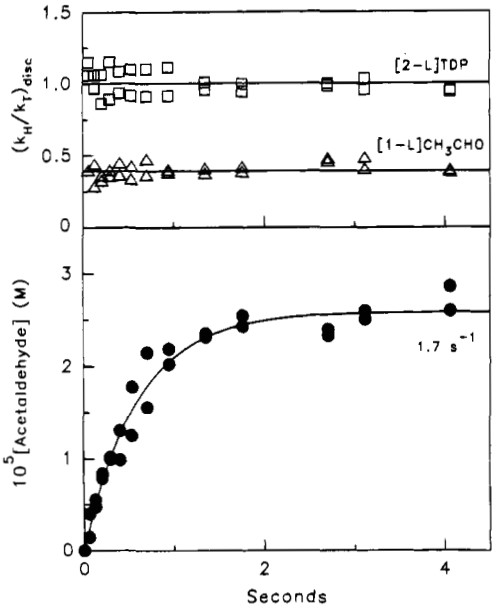


FIGURE 3: Solvent discrimination tritium isotope effect, $(k_H/k_T)_{disc}$, for C(2)-H→T transfer to PDC-bound TDP and C(α)-H→T transfer to PDC-bound HETDP under single-turnover conditions. Lower panel: formation of acetaldehyde catalyzed by 25 μM pyruvamide-activated holo-PDC (6.0 mg mL^{-1}) at 30 °C in [^{3}H]H_2O (0.85 Ci mol^{-1}) containing 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 1.7 s^{-1}. Upper panel: dependence of $(k_H/k_T)_{disc}$ for C(2)-H→T transfer to PDC-bound TDP (□) and C(α)-H→T transfer to PDC-bound HETDP (△) on the extent of the single-turnover reaction. The lines represent the mean values of $(k_H/k_T)_{disc}$.

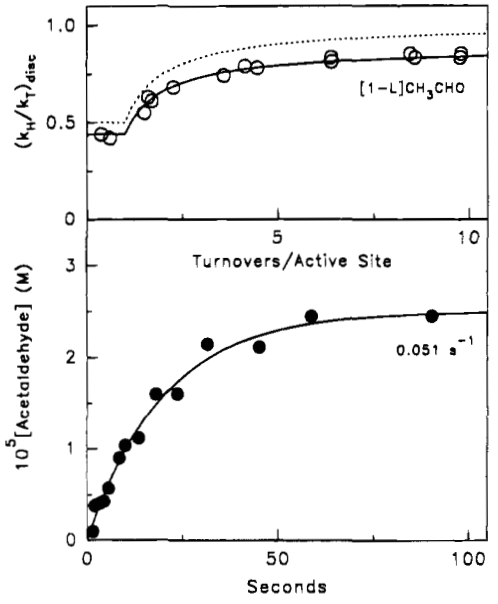


FIGURE 4: Solvent discrimination tritium isotope effect, $(k_H/k_T)_{disc}$, for C(α)-H→T transfer to PDC-bound HETDP under transient steady-state conditions. Lower panel: formation of acetaldehyde catalyzed by 0.625 μM pyruvamide-activated holo-PDC (0.15 mg mL^{-1}) at 30 °C in [^{3}H]H_2O (0.85 Ci mol^{-1}) containing 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM TDP, 10 mM $MgSO_4$, and 25 μM sodium pyruvate (●). The line is drawn for a first-order rate constant of 0.051 s^{-1}. Upper panel: dependence of $(k_H/k_T)_{disc}$ for C(α)-H→T transfer to PDC-bound HETDP (and subsequent formation of [1-L]acetaldehyde) on the extent of the transient steady-state reaction of 25 μM sodium pyruvate (○). The solid line is described by eq 2 using $\phi = 0.88 \pm 0.06$ (see text); the dashed line is drawn for $\phi = 1$.

acetaldehyde was recovered for HPLC analysis as acetaldehyde (2,4-dinitrophenyl)hydrazone. The value of $k_{obsd} = 1.7$ s^{-1} obtained at 25 μM PDC agrees with the value calculated by numerical integration with previously reported microscopic rate constants for the reaction of activated PDC and pyruvate (Alvarez et al., 1991) using the program KINSIM (Barshop et al., 1983; Crane et al., 1993). The agreement between the observed and calculated rate constant provides additional evidence that incubation of PDC with pyruvamide, before substrate addition and the onset of t, rapidly and completely activates PDC (Hübner et al., 1978, 1988). This shows that activation of PDC is not rate limiting under these experimental conditions. Pyruvamide does not compete with pyruvate at the active site but binds elsewhere on the protein as an allosteric effector (Hübner et al., 1978).

Figure 3 (upper panel) shows the dependence of the solvent discrimination tritium isotope effect, $(k_H/k_T)_{disc}$, for C(2)-H→T exchange in PDC-bound [thiazole-2-H]TDP and C(α)-H→T exchange in PDC-bound HETDP on the extent of reaction for [1-L]acetaldehyde (L = H or T) formation catalyzed by 25 μM pyruvamide-activated PDC at 30 °C in [^{3}H]H_2O containing 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM $MgSO_4$, and 25 μM sodium pyruvate under single-turnover conditions. The isotopic composition of PDC-bound [thiazole-2-L]TDP was independent of the extent of the reaction and not distinguishable from the isotopic composition of the solvent with a value of $(k_H/k_T)_{disc} = 1.0 \pm 0.1$ ($N = 26$). The isotopic composition of [1-L]acetaldehyde (derived from PDC-bound [α-L]-HETDP) was also independent of the extent of the reaction with $(k_H/k_T)_{disc} = 0.39 \pm 0.05$ ($N = 26$). It was previously demonstrated that T→H exchange from electronegative atoms on TDP, HETDP, substrate, and buffer components in the acid-quenched reaction solutions is instantaneous and complete on the $0.06 < t < 4$ s time scale (Washabaugh & Jencks, 1989; Stivers & Washabaugh, 1992; Crane et al., 1993). Little (≤8.7%) or no C(2)-hydron exchange occurs from PDC-bound TDP in the absence of substrate during the $0.06 < t < 4$ s time course under these reaction conditions.

The kinetics of [1-L]acetaldehyde (L = H or T) formation catalyzed by pyruvamide-activated PDC containing [thiazole-2-H]TDP at 30 °C in [^{3}H]H_2O containing 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM TDP, 10 mM $MgSO_4$, and 25 μM sodium pyruvate were also followed by HPLC under transient steady-state conditions. Figure 4 (lower panel) shows that conversion of 25 μM sodium pyruvate to [1-L]acetaldehyde catalyzed by 0.625 μM pyruvamide-activated PDC (2.5 μM active sites) follows a single exponential ($k_{obsd} = 0.051$ s^{-1}) with no evidence of a lag or "burst" kinetics. T→H exchange involving enolization of pyruvate is slow ($t_{1/2} > 8$ days) (Alvarez et al., 1991) so this reaction does not interfere with measurements on the $0.06 < t < 50$ s time scale.

Figure 4 (upper panel) shows the dependence of the solvent discrimination tritium isotope effect, $(k_H/k_T)_{disc}$, for C(α)-H→T exchange in PDC-bound HETDP on the extent of reaction for [1-L]acetaldehyde (L = H or T) formation catalyzed by 0.625 μM pyruvamide-activated PDC at 30 °C in [^{3}H]H_2O containing 100 mM sodium MES buffer (pH 6.00), 100 mM pyruvamide, 10 mM TDP, 10 mM $MgSO_4$, and 25 μM sodium pyruvate under transient steady-state conditions. [1-L]Acetaldehyde formation under transient

steady-state ([E] < [S]) conditions shows solvent discrimination tritium isotope effects that increase over the range $(k_H/k_T)_{disc} = 0.39 \pm 0.05$ (single turnover) to 0.86 (ten turnovers) ($N = 15$). The relationship between the isotopic composition of [1-L]acetaldehyde product, $(k_H/k_T)_{disc}$, and the fractionation factor, ϕ, for the group(s) that protonate(s) the C(α) position in PDC-bound HETDP may be described by eq 2 (derived

$$(k_H/k_T)_{disc} = \frac{\phi(x - 1) + \phi/2}{x} \quad (2)$$

empirically from these data), where x is the number of PDC turnovers. Because [1-L]acetaldehyde is formed after specific protonation of HETDP C(α)-L by a catalytic group(s) at equilibrium (ϕ_1) with solvent-derived T^+ and PDC-bound [thiazole-2-L]TDP-derived H^+ ($x \leq 1$) or T^+ ($x > 1$) (see below), a minimum value of $(k_H/k_T)_{disc} = \phi/2$ is observed for [1-L]acetaldehyde formed in the first turnover ($x = 1$) and a maximum value of $(k_H/k_T)_{disc} = \phi$ is observed after many turnovers ($x > 10$). The observed dependence of $(k_H/k_T)_{disc}$ for [1-L]acetaldehyde formation on the extent of the reaction is described by eq 2 using $\phi = 0.88 \pm 0.06$: the dashed line in Figure 4 (upper panel) is drawn for $\phi = 1$.

DISCUSSION

Fractionation Factors for TDP and HETDP. Fractionation factors, ϕ, are useful in investigations of solvent isotope effects on enzymic proton transfer to and from carbon. Fractionation factors measure the tendency of a solute site to fractionally contain L^+ (L = D or T),[2] compared to the L fraction of the solvent, and reflect the stiffness or tightness of binding of a solute site compared to the O−L sites of solvent (see eq 1). L^+ accumulates where binding is tighter at a site ($\phi > 1$), and H^+ accumulates where binding is looser at a site ($\phi < 1$); by definition $\phi = 1$ for solvent. A tighter site is one where intramolecular or intermolecular restraints render motion more energy demanding— a solute site may be tighter than the average site in the solvent because the solute has one or more isotopically sensitive vibrations that are of higher frequency (and, consequently, have larger force constants) than those of solvent.

The functional groups that are involved in enzymic acid−base and nucleophilic catalysis (ROH, RCO_2H, N−H bonds) have reactant-state fractionation factors near unity (Schowen & Schowen, 1982). Consequently, reactant-state fractionation of these enzymic functional groups does not contribute significantly to equilibrium or kinetic isotope effects, and equilibrium solvent isotope effects for reversible proton transfer steps that involve these functional groups are near unity. The S−H bond of cysteine is an exception among enzyme functional groups with $\phi = 0.55 \pm 0.1$ (Schowen & Schowen, 1982). Low fractionation factors in the range $\phi = 0.3-0.6$ can also reflect strong H bonds of the types O···H···O and N···H···O in which the pK_a values of the H-bond donor and the conjugate acid of the acceptor are closely matched (Cleland & Kreevoy, 1994). Medium effects usually do not contribute significantly to equilibrium solvent isotope effects (Quinn & Sutton, 1991); however, the strength of hydrogen bonds ($\phi < 1$) is enhanced by the exclusion of water (Cleland & Kreevoy, 1994).

The fractionation factor for both nonenzymic C(2)-hydron exchange in TDP (**1a**) and C(α)-hydron exchange in HETDP (**2a**) was shown to be unity (Figures 1 and 2) and independent of medium effects (ionic strength or organic solvent) or pK_a of the carbon acid (Tables 1 and 2). The fractionation factor of $\phi_{C(2)} = 0.98 \pm 0.06$ for C(2)-L in 3-R-4-methylthiazolium ions, including TDP, is in the upper range of (H−D) fractionation factors of $\phi = 0.84 \pm 0.06$ that are usually observed for sp^2 C−H bonds at 25 °C (Kresge et al., 1987). Both the directly measured ($\phi_{C(\alpha)} = 1.01 \pm 0.07$) and calculated ($\phi = 1.1 \pm 0.1$)[4] values for C(α)-L in 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions, including HETDP, are consistent with (H−D) fractionation factors for other tertiary sp^3 C−H bonds in the range $\phi = 1.09 \pm 0.11$ at 25 °C (Kresge et al., 1987). We conclude that reactant-state fractionation of the C(2) position in PDC-bound TDP and C(α) position in PDC-bound HETDP does not contribute significantly to observed isotopic discrimination (and equilibrium solvent isotope effects) during catalysis of acetaldehyde formation by PDC: there is no intrinsic preference for H^+ or T^+. The lack of equilibrium solvent isotope effects for hydron transfers to and from C(2) of TDP and C(α) of HETDP involving carboxyl-, amino-, and hydroxyl-containing catalysts greatly simplifies the analysis of multistep enzymic reactions involving these thiamin-derived carbon acids.

A fractionation factor of $\phi < 1$ consistent with a low-barrier hydrogen bond for enzymic C(2)- and C(α)-hydron transfer is unlikely. There is no strong hydrogen bond between water ($pK_a = -1.74$) or less acidic groups and the *highly localized* carbanion in the C(2)-ylide ($pK_a \leq 18$) in either the ground or transition state that could contribute significant transition-state stabilization to C(2)-hydron transfer (Washabaugh & Jencks, 1988). The conclusion that transition-state stabilization by hydrogen bonding is not significant in C(2)-hydron transfer implies that hydrogen bonding is also not likely to contribute significant stabilization to the ground or transition state of C(α)-hydron transfer to form a *delocalized* carbanion. There is little, if any, solvent isotope effect for diffusion of T^+ in L_2O; the solvent isotope effect on diffusion of HTO in H_2O and DTO in D_2O is small (≤3%) (Weingärtner, 1982) and would not be expected to be significantly larger if LTO is hydrogen bonded to L_3O^+.

The fractionation factors of the exocyclic 4′-amino group (Ar−NH_2) of free TDP and HETDP (Ar−NH_3^+ ↔ H^+ + Ar−NH_2; $pK_a \approx 0$) and the 4′-imino group (Ar−NH^-) in N(1′)-protonated TDP and HETDP (Ar−NH_2 ↔ H^+ + Ar−NH^-; $pK_a \approx 12$) (Washabaugh & Jencks, 1988) are also of interest. Several workers have proposed that the 4′-amino group (Golbik et al., 1991) and the 4′-imino group (Jordan & Mariam, 1978; Ermer et al., 1992) participate in general acid−base catalysis of C(2)- and C(α)-proton transfer in TDP-dependent enzymes. Fractionation factors for the exocyclic 4′-amino and -imino group have not been measured directly because of technical limitations. However, the (H−T) fractionation factors[5] for the exchangeable N−H bonds in *p*-nitroaniline (*p*-NO_2-$C_6H_4NH_2$) and its *N*-methylated analogs (*p*-NO_2-C_6H_4NHMe and *p*-NO_2-$C_6H_4N(+)HMe_2$) are in the range $\phi = 1.3-1.5$, which suggests $\phi = 1.43$ for Ar−NH_2 and $\phi = 1.35$ for Ar−NH_3^+ (Gold & Tomlinson, 1971). The (H−T) fractionation factor of $\phi = 1.18$ for the N−H bond in *N*-methylacetamide has been used previously as an

[5] Tritium effects are the 1.442 power of deuterium effects (Swain et al., 1958; Cleland, 1980).

estimate of ϕ for Ar−NH^- (Quinn & Sutton, 1991). We conclude that $\phi \geq 1.2$ for the exocyclic 4′-amino or -imino group in free and PDC-bound TDP and HETDP. The aminopyrimidinyl group has no effect, other than inductive, on the nonenzymic C(2)-proton exchange rate of TDP (Washabaugh & Jencks, 1988) or the C(α)-proton exchange rate of HETDP (Stivers & Washabaugh, 1992).

Solvent-Derived Protons. The value of $(k_H/k_T)_{disc} = 1.0$ for reprotonation of TDP C(2)-L (Figure 3) suggests that hydron transfer to C(2) upon carbon−carbon bond cleavage and product release is not rate-limiting and occurs either from solvent directly or through the mediation of a catalytic acid at equilibrium with bulk solvent with a fractionation factor of unity. 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 in the presence (Crane et al., 1993) or absence of substrate (Harris & Washabaugh, 1995). This also provides evidence against rate-limiting C(2)-hydron transfer between C(2)-L in PDC-bound TDP and a catalytic base with $-7 \leq \Delta pK_a$ ($= pK_a^{BH} - pK_a^{C(2)\text{-}L}$) ≤ 7 (Crane et al., 1993). Asymmetry in the transition state for C(2)-hydron transfer (a change in transition-state structure as the energy of the hydron donor or the base is changed) results in suppression of the intrinsic primary kinetic isotope effect, $(k_H/k_T)_{int} = 23$ at $\Delta pK_a = 0$, and is consistent with a small barrier for C(2)-L abstraction that is similar to that for the electronegative atoms of "normal" acids.

Approximately 40% of the tritium label is found at the C(α) position of PDC-bound HETDP after a single PDC turnover regardless of whether the label originates in the solvent (Figures 3 and 4) or at the C(2) position of PDC-bound TDP (Harris & Washabaugh, 1995). There is little (≤3%) or no return of the abstracted C(2)-hydron to the C(2) position of PDC-bound TDP (Harris & Washabaugh, 1995). We conclude that C(α)-hydron transfer to form PDC-bound HETDP involves competition between the abstracted C(2)-hydron and another hydron originating from solvent. *Partial* exchange of the C(2)-derived hydron into solvent also requires that (i) hydron transfer from C(2) occurs to a catalytic base in which the conjugate catalytic acid is shielded from hydron exchange with *bulk* solvent, (ii) the conjugate catalytic acid is involved in transfer of the C(2)-derived hydron to the C(α) position of HETDP, and (iii) C(2)-hydron transfer to regenerate the coenzyme occurs either from solvent directly or from a second catalytic acid of the enzyme that is equilibrated with the bulk solvent ($\phi = 1.0$).

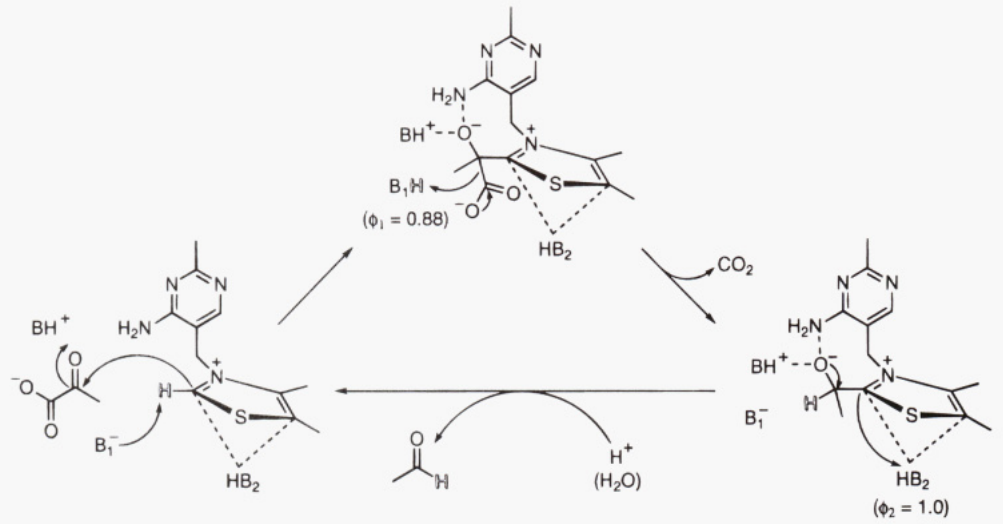
The 2-fold increase in the value of $(k_H/k_T)_{disc}$ for the [1-L]-acetaldehyde product under steady-state (Figure 4) compared to single-turnover conditions (Figure 3) is consistent with a fractionation factor of $\phi_1 = 0.88$ for the residue(s) involved in C(α)-hydron transfer to form PDC-bound HETDP (Scheme 1). The experimental observations are that a value of $(k_H/k_T)_{disc} = \phi/2$ is observed for [1-L]acetaldehyde formed in the first turnover (Figure 3) and a limiting value of $(k_H/k_T)_{disc} = \phi$ is observed after many (>10) turnovers (see eq 2) (Figure 4). These results require a mechanism in which [1-L]-acetaldehyde is formed after specific protonation of HETDP C(α)-L by a catalytic group or groups at equilibrium (ϕ_1) with solvent-derived T^+ and PDC-bound [thiazole-2-L]TDP-derived H^+ (single turnover) or T^+ (multiple turnovers). This supports the conclusion that catalysis of acetaldehyde formation by PDC involves specific protonation of both HETDP C(α)-L and TDP C(2)-L ($\phi_2 = 1.0$, Scheme 1) and requires at least two catalytic groups. The (H−T) fractionation factor of $\phi_1 = 0.88 \pm 0.06$ confirms and extends the observation of 44 ± 2% incorporation of D into [1-L]acetaldehyde during catalysis by PDC in 50:50 H_2O−D_2O under steady-state conditions (Ermer et al., 1992), which corresponds to a (H−T) fractionation factor of $\phi = 0.83 \pm 0.04$ [$=(0.44/0.50)^{1.442}$] for protonation of HETDP C(α)-L.[5]

The equilibrium involving the hydron that ends up at C(α) may represent (i) 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, (ii) 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 (iii) 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. The values of $\phi \leq 1$ for enzymic hydron transfer reactions involving TDP C(2)-L and HETDP C(α)-L provide no evidence that the exocyclic 4′-amino or -imino group ($\phi \geq 1.2$) contributes significant intramolecular general acid−base catalysis in the enzyme-bound coenzyme.

Proposed Mechanism. In this and the preceding paper in this issue (Harris & Washabaugh, 1995) we describe an examination of C(2)-hydron transfer to and from PDC-bound [thiazole-2-L]TDP (L = H or T) and C(α)-hydron transfer to PDC-bound HETDP in isotopically labeled solvent. We have determined the distribution (solvent and product) of the C(2)-proton derived from TDP and the source of catalytic protons for protonation of the C(2) position in TDP and the C(α) position in HETDP (solvent and cofactor, respectively). The combined results from this work and the preceding paper may be described by the proposed mechanism shown in Scheme 3.

Figure 5A shows the enzyme catalytic groups that are potentially close enough to interact with PDC-bound TDP and HETDP at the active site. The closest catalytic groups that are solvent accessible include the carboxylates of Asp-28 and Glu-477, the imidazole of His-115, and the exocyclic 4′-amino group of TDP. We propose that B_1 and HB_2 in Scheme 3 are Asp-28 and Glu-477, respectively, and that His-115 and the aminopyrimidinyl group of TDP interact with the alcoholate anion of enzyme-bound α-lactyl-TDP and HETDP. The side-chain functional groups of His-115 and Asp-28 are each positioned 5−6 Å from the C(2) position and approximately 3−4 Å from the C(α) position of HETDP. The carboxylate of Glu-477 is located approximately 4 Å perpendicular to the plane of the thiazolium ring. Important conformational changes associated with regulatory sulfhydryl addition−elimination reactions could result in the proper positioning of Asp-28 for C(2)-hydron abstraction and subsequent hydron transfer to form HETDP (Baburina et al., 1994; Alvarez et al., 1995). The bell-shaped pH dependence of k_{cat} (Green et al., 1941; Singer & Pensky, 1952; Schellenberger et al., 1967; Jordan et al., 1978) provides evidence for at least one unprotonated ($pK_a = 5.3$−5.8) and one protonated ($pK_a = 5.8$−6.3) catalytic group at the active site. In nonaqueous and apolar microenvironments [such as the active site (see below)] the pK_a values for neutral acids such as the carboxyl groups of Asp-28 and Glu-477

Scheme 3



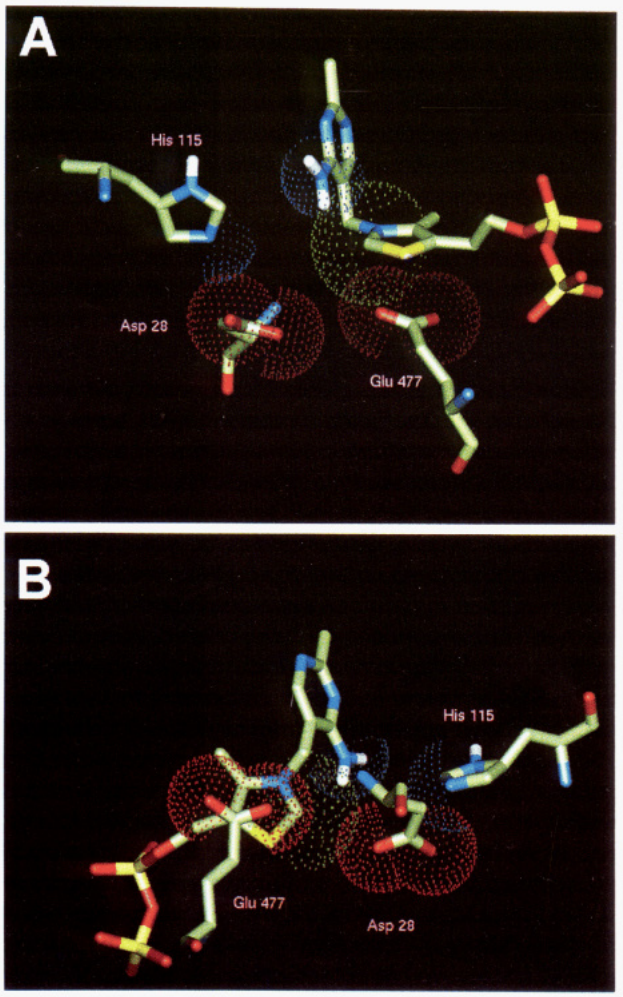


FIGURE 5: (A) Potential active-site catalytic residues (His-115, Asp-28, and Glu-477) in yeast pyruvate decarboxylase (EC 4.1.1.1). (B) View of Glu-477 from below the thiazolium ring of TDP. Van der Waals radii are indicated. These pictures were generated using the program QUANTA from the crystal structure in the absence of substrate (Dyda et al., 1993).

can be elevated (Kokesh & Westheimer, 1971).

Several groups have evaluated the rate of C(2)-hydron transfer from TDP and found that the enzyme-mediated reaction of thiamin with pyruvate is at least $10^{3.5}$ times faster than the maximum rate possible in the nonenzymic reaction. The scale of this ratio suggests that a major role of TDP-dependent enzymes might be to decrease $pK_a^{C(2)-L}$ of enzyme-bound TDP to a value of ≤ 14.5 ($=18 - 3.5$) in the presence of substrate (Crosby & Lienhard, 1970; Kemp & O'Brien, 1970; Washabaugh & Jencks, 1988). 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 attributed the 4-fold rate enhancement of C(2)-hydron exchange in PDC-bound TDP to a small decrease in the pK_a value for C(2)-H at the active site to 17.5 (Harris & Washabaugh, 1995).

An alternate explanation for the relatively fast enzymic proton transfer was offered by several groups who proposed that the aminopyrimidinyl group functions as an intramolecular catalyst for C(2)-hydron abstraction (Golbik et al., 1991) and hydron transfer to the C(α) position of enzyme-bound HETDP (Ermer et al., 1992). As discussed above, the magnitudes of isotopic discrimination in the PDC-catalyzed reaction of TDP with pyruvate under single-turnover and transient steady-state conditions in $[^3H]H_2O$ provide no evidence that the aminopyrimidinyl group contributes significant intramolecular catalysis for hydron transfer to and from enzyme-bound TDP C(2)-L or HETDP C(α)-L but do not strictly exclude involvement of the aminopyrimidinyl group. The (H−T) fractionation factor of $\phi_1 = 0.88$ for the residue(s) involved in C(α)-hydron transfer to form HETDP may reflect exclusion of water in the active site, since a nonprotic environment favors the formation of strong hydrogen bonds with $\phi < 1$ (Cleland & Kreevoy, 1994). The TDP binding site of yeast pyruvate decarboxylase is apolar (Wittorf & Gubler, 1970; Dyda et al., 1993), and the susceptibility of TDP species toward covalent hydration requires that enzyme-bound TDP must be shielded from bulk water (Gruys et al., 1989; Washabaugh et al., 1993). However, hydrogen bonding of solvent to TDP-derived carbanions, if present, is very weak (Washabaugh & Jencks, 1988), and strong hydrogen bonding in the transition state is not necessary for rapid proton transfer to and from carbon.

Involvement of the exocyclic 4′-amino group in the catalytic mechanism of PDC was suggested by others, in part because elimination or substitution of the 4′-amino group

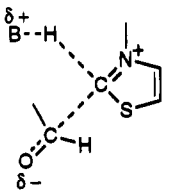
and elimination of the N(1′) atom of the pyrimidine ring produced TDP analogs that were inactive as coenzymes (Schellenberger, 1967) and because the 4′-amino group is near the C(2) position in enzyme-bound TDP (Figure 5) (Dyda et al., 1993). These results do not exclude the possibility that noncovalent interactions between PDC and the aminopyrimidinyl group of TDP are important for activation of catalysis (Washabaugh et al., 1993) and structural perturbations in the aminopyrimidinyl group have deleterious effects on these important enzymic interactions.[6] Direct evidence against proposals that the aminopyrimidinyl group of PDC-bound TDP is an *essential* intramolecular general acid−base catalyst includes the observation of decarboxylation, but no acetaldehyde formation, in the reaction of pyruvate catalyzed by PDC-bound oxyTDP (in which the 4′-amino group is replaced by a carbonyl group) (Schellenberger, 1967).

We suggest that the exocyclic 4′-amino group is involved in the stabilization and destabilization of the alcoholate anion of enzyme-bound α-lactyl-TDP and HETDP, respectively, necessary for product formation and release (Scheme 3). The failure to detect acetaldehyde in the reaction of PDC-bound oxyTDP with pyruvate (Schellenberger, 1967) suggests accumulation of a stable, enzyme-bound intermediate after decarboxylation in the absence of the 4′-amino group. Buffer base-catalyzed cleavage of 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions, including HETDP, to the corresponding aldehyde and thiazolium ion in aqueous solution was formulated as general acid catalysis of the departure of thiazolium ion from the alcoholate anion of the substrate (general base catalysis of thiazolium ion attack on the aldehyde in the reverse direction) (Crane & Washabaugh, 1992). Schellenberger previously suggested a N···H···O hydrogen bond between the exocyclic 4′-amino group and the α-hydroxyl group in HETDP based, in part, on kinetic experiments with PDC-bound [4-aminopyrimidine-4-^{15}N]-TDP (Schellenberger, 1982).

One of the several mechanisms that are utilized by TDP-dependent enzymes to catalyze aldol-type reactions may involve assistance to proton removal by interaction with an electrophile in the transition state and assistance to carbon−carbon bond cleavage and formation by a significant amount of proton transfer in the transition state (Crane & Washabaugh, 1992). This mechanism would avoid the formation of the relatively unstable C(2)-ylide as a discrete intermediate and the transition state leading to its formation (Washabaugh & Jencks, 1988). Enzymic stabilization of the C(2)-ylide (or destabilization of TDP) in a concerted mechanism could serve to facilitate proton transfer rather than simply increase the lifetime of the C(2)-ylide for a stepwise mechanism.

We have measured the effects of catalyst acidity, leaving group (thiazolium ion) basicity, and increasing the energy of the electrophile on the rate of retrograde aldol-type reactions of 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions, including HETDP, and have estimated the interactions between these substituent effects (Crane & Washabaugh, 1992). The signs of the interaction coefficients that describe the changes in the substituent effects provide evidence for coupling between proton donation and leaving group expulsion; they confirm a concerted mechanism for these model elimination reactions in aqueous solution. The results support the idea that stepwise and concerted mechanisms can coexist for substitution at carbon involving a moderately unstable nucleophile derived from a "normal" carbon acid.

To our knowledge this was the first demonstration of a reaction involving a carbanion "intermediate" following a concerted mechanism when the intermediate of a stepwise mechanism has a significant lifetime and is one of the first examples of a "nephotic" transition state (Liu et al., 1993) (HB_2 in Scheme 3). There is precedent for a π-complex between an aromatic ring and a hydrogen bond donor (an "Hπ bond" or "on-face" H-bond) that may be relevant [see, for example, Liu et al. (1993)]. Glu-477 is positioned well for the formation of an "on-face" H-bond between the proton and the π electrons of the thiazolium ring (Figure 5B). Although overlap with antibonding σ^* orbitals is possible, more conventional resonance delocalization is unlikely to be important for the electron pair involved in C(2)-hydron transfer (Washabaugh & Jencks, 1988). Acid catalysis involving front-side electrophilic displacement at the thiazolium C(2)−C(α) bond with a transition state in which the leaving C(α) and entering proton interact with C(2)



is unlikely because there is no clear precedent for such electrophilic assistance with these steric requirements (Washabaugh & Jencks, 1989).

It is also not known whether the C(α)-carbanion/enamine derived from HETDP exists as a discrete intermediate on PDC (Stivers & Washabaugh, 1993). The magnitudes of primary $^{L}(V/K)$-type (L = D or T) isotope effects on C(α)-proton transfer from PDC-bound HETDP provide no evidence for significant breakdown of the Swain−Schaad relationships (Stivers & Washabaugh, 1993) that would indicate partitioning of the putative C(α)-carbanion/enamine intermediate between HETDP and products. A (1.10 ± 0.02)-fold ^{14}C isotope discrimination against [1,2-^{14}C]-acetaldehyde in acetoin formation is inconsistent with a stepwise mechanism, in which the addition step occurs after rate-limiting formation of the C(α)-carbanion/enamine as a discrete enzyme-bound intermediate, and provides evidence for a concerted reaction mechanism with an important component of carbon−carbon bond formation in the transition state. The mechanism of C(α)-carbanion/enamine addition to carbonyl compounds in aqueous solution has not been investigated in detail.

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[6] Studies of the reactivity of PDC with small TDP analogs, 3,4-dimethyl-5-(2-pyrophosphoethyl)thiazolium ion (DMT) and 4-amino-2-methylpyrimidine (AP), suggest that noncovalent interactions between the enzyme and these functional domains of TDP are responsible for a rate acceleration of $10^{8.6}$, which is a substantial portion of the total rate acceleration of $10^{12.6}$ caused by the enzyme (Alvarez et al., 1991). Approximately 75% (3.2 kcal mol^{-1}) of the coenzyme-induced destabilization energy in this system can be caused by binding of the two separate functional domains (DMT and AP), and only 25% (1.2 kcal mol^{-1}) can be attributed to a requirement for the different parts of TDP to be covalently linked (Vaccaro and Washabaugh, unpublished results).

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