Catalysis of Acetoin Formation by Brewers' Yeast Pyruvate Decarboxylase Isozymes†

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ABSTRACT: Catalysis of C(α)-proton transfer from 2-(1-hydroxyethyl)thiamin diphosphate (HETDP) by pyruvate decarboxylase isozymes (PDC; EC 4.1.1.1) from *Saccharomyces carlsbergensis* was investigated by determining the steady-state kinetics of the reaction of [1-L]acetaldehyde (L = H, D, or T) to form acetoin and the primary kinetic isotope effects on the reaction. The PDC isozyme mixture and α_4 isozyme (α_4-PDC) have different steady-state kinetic parameters and isotope effects for acetoin formation in the presence and absence of the nonsubstrate allosteric effector pyruvamide: pyruvamide activation occurs by stabilization of the acetaldehyde/PDC ternary complex. The magnitudes of primary $^{L}(V/K)$-type (L = D or T) isotope effects on C(α)-proton transfer from α_4-PDC-bound HETDP provide no evidence for significant breakdown of the Swain–Schaad relationship that would indicate partitioning of the putative C(α)-carbanion/enamine intermediate between HETDP and products. The substrate concentration dependence of the deuterium primary kinetic isotope effects provides evidence for an intrinsic isotope effect of 4.1 for C(α)-proton transfer from α_4-PDC-bound HETDP. 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.

Pyruvate decarboxylase (PDC)[1] (2-oxo-acid carboxy-lyase; EC 4.1.1.1) is a thiamin diphosphate (TDP, **1**) dependent enzyme that catalyzes the irreversible nonoxidative decarboxylation of pyruvate to form acetaldehyde (Scheme I, upper pathway) (Alvarez et al., 1991). PDC also catalyzes an aldol-type condensation reaction between two molecules of acetaldehyde to form the α-ketol acetoin (**2**) (Scheme I; lower pathway) (Chen & Jordan, 1984). The C(α)-carbanion/enamine (**3**) derived from 2-(1-hydroxyethyl)thiamin diphosphate (HETDP, **4**) has been implicated in aldol-type addition reactions between HETDP and carbonyl compounds catalyzed by several TDP-dependent enzymes (Kluger, 1992). Conjugated C(α)-enamines have been observed as intermediates during catalysis by PDC of decarboxylation and other reactions involving (*E*)-2-oxo-4-(4-R-phenyl)-3-butenoic acids that have strong electron-withdrawing substituents on the phenyl ring (Zeng et al., 1991).

The lifetimes of intermediates determine whether elimination and other reactions of carbanions can proceed through a stepwise mechanism; if the intermediate is too unstable to exist, the reaction must be concerted. A pK_a value of ≤15 is required for C(α)-H on PDC-bound HETDP if the C(α)-carbanion/enamine exists as an intermediate in a stepwise enzymatic mechanism for acetoin formation (Scheme I, lower pathway).[2] The pK_a values in the range 18.4–19.8 for C(α)-H on HETDP in aqueous solution show that the C(α)-carbanion/enamine is quite unstable in aqueous solution (Stivers & Washabaugh, 1992). However, rate-limiting diffusion-controlled protonation and deprotonation in this system mean that there is internal return[3] in C(α)-proton exchange and

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[1] Abbreviations: PDC, pyruvate decarboxylase; TDP, thiamin diphosphate; HETDP, 2-(1-hydroxyethyl)thiamin diphosphate; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; Girard's reagent P, 1-(carboxymethyl)pyridinium chloride hydrazide; Me_2SO, dimethyl sulfoxide; ADH, alcohol dehydrogenase; NADH (NAD^+), reduced (oxidized) nicotinamide adenine dinucleotide; HET, 2-(1-hydroxyethyl)-thiamin.

[2] The upper limit for the pK_a of PDC-bound HETDP was calculated according to the following equation at pH 6.00.

$$-\overset{|}{\underset{|}{C}}\cdot H + B \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} -\overset{|}{\underset{|}{C}}^{-} + HB^+$$

The value of $k_a/k_{-a} = 10^{-12}$ was calculated by assuming pK_a = 7 for the base at the active site, p$K_a \approx 19$ for C(α)-H on HETDP in aqueous solution (Stivers & Washabaugh, 1992), and the equation $\log k_a/k_{-a} = pK_a^{BH} - pK_a^{C(\alpha)\text{-}H}$. A value of $k_{-a} = 3 \times 10^9$ M^{-1} s^{-1} was assumed for the diffusion-controlled reaction in the reverse direction (Stivers & Washabaugh, 1992), which gives $k_a = 3 \times 10^{-3}$ M^{-1} s^{-1} = (10^{-12})(3×10^9 M^{-1} s^{-1}). At pH 6.00 the enzymatic catalyst is ≈10% fraction base, and for a standard state of 1.0 M at the active site, this corresponds to 0.1 M catalyst and $k_a = 3 \times 10^{-4}$ s^{-1} = (0.1 M)(3×10^{-3} M^{-1} s^{-1}) at pH 6.00. The turnover number of PDC for acetoin formation is ≈4 s^{-1} (Table I). We conclude that PDC must catalyze C(α)-H abstraction from HETDP by at least $1.3 \times 10^4 = 4$ s^{-1}/3×10^{-4} s^{-1} if the C(α)-carbanion/enamine exists as an intermediate in a stepwise enzymatic addition mechanism. Because C(α)-H removal occurs at the maximum possible rate for a given equilibrium constant, this ≥10^4 enzymatic acceleration corresponds to $pK_a^{C(\alpha)\text{-}H} \leq 15 = 19 - \geq 4$ for PDC-bound HETDP.

[3] Rates of deprotonation of carbon acids in solvents which are considerably more acidic than the acids themselves are subject to the effects of "internal return". When both proton transfer (k_1) and diffusion-controlled separation of the products (k_2) are partially rate limiting, reprotonation of the hydrogen-bonded carbanion (k_{-1}) competes with k_2 and the carbanion intermediate partitions between products and reactants (Washabaugh & Jencks, 1989): k_{-1}/k_2 gives the extent of internal return, which is analogous to a "commitment factor" [see, for example, Cleland (1991)].

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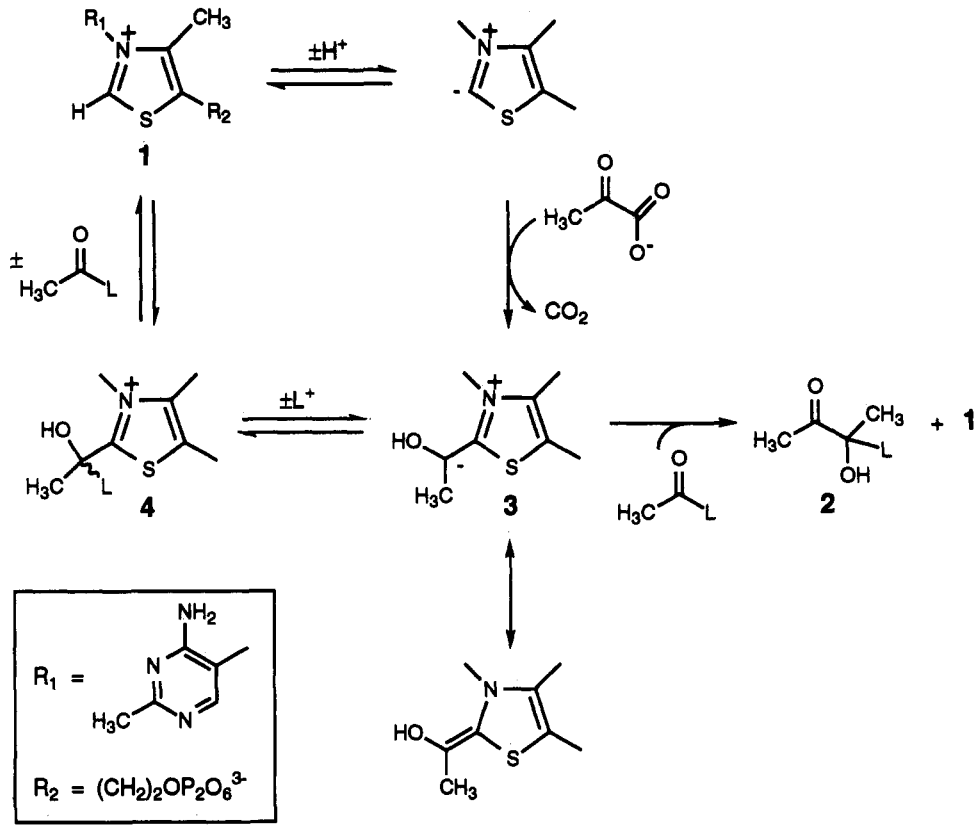
Scheme I



prove that C(α)-carbanion/enamines can have a significant lifetime in aqueous solution (Stivers & Washabaugh, 1992). This means PDC either changes the relative thermodynamic stabilities of HETDP and its C(α)-carbanion/enamine or provides a one-step, concerted pathway that avoids such an unstable carbanion intermediate. The stepwise pathway must exist, but it might be bypassed by a concerted pathway because of the relative instability of the carbanion (Crane & Washabaugh, 1992; Thibblin & Jencks, 1979). We are interested in how PDC overcomes kinetic barriers for C(α)-proton transfer from HETDP and whether PDC stabilizes or avoids the C(α)-carbanion/enamine derived from HETDP.

Analysis of enzymatic and nonenzymatic kinetic isotope effects provides details about the relative kinetic significance of sequential transition states. Streitwieser and co-workers (Streitwieser et al., 1971, 1973) showed that, for the base-catalyzed exchange of C–L bonds (where L = H, D, or T),[4] the amount of partitioning of the carbanion intermediate between reactants and products (internal return) could be measured from the breakdown of the Swain–Schaad relation (eq 1) (Swain et al., 1958), which relates the tritium and

$$\log(k_H/k_T)_{obsd} = 1.442 \times \log(k_H/k_D)_{obsd} \quad (1)$$

deuterium isotope effects; internal return for C(2)-proton exchange from thiamin (**1**, $R_2 = (CH_2)_2OH$) in aqueous solution was demonstrated using this technique (Washabaugh & Jencks, 1989). Deviations from eq 1 occur when two or more transition states are rate limiting, and Northrop has shown that this breakdown of eq 1 can be used to examine the details of enzymatic reactions (Northrop, 1975, 1982). We have used this method to obtain evidence against significant partitioning of the C(α)-carbanion/enamine intermediate between HETDP and products during catalysis of acetoin formation by PDC.

In this paper we describe evidence that confirms and extends a previous conclusion that C(α)-proton transfer is at least partially rate limiting in the reaction to form acetoin (Chen & Jordan, 1984). The magnitudes of primary $^{L}(V/K)$-type (L = D or T) isotope effects on C(α)-proton transfer from PDC-bound HETDP provide evidence (1) against significant breakdown of the Swain–Schaad relationship that would indicate significant internal return of the hydron; (2) for an intrinsic isotope effect, ^{D}k, of 4.1 for C(α)-proton transfer; (3) that PDC isozymes have different steady-state kinetic parameters and isotope effects for acetoin formation in the presence and absence of the nonsubstrate allosteric effector pyruvamide; and (4) that pyruvamide activation occurs by stabilization of the acetaldehyde/PDC ternary complex. A (1.10 ± 0.02)-fold ^{14}C isotope discrimination against [1,2-^{14}C]acetaldehyde in acetoin formation is inconsistent with a stepwise reaction mechanism, in which the addition step occurs after rate-limiting formation of the C(α)-carbanion/enamine as a discrete enzyme-bound intermediate.

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). 2-(1-Hydroxyethyl)-3,4-dimethylthiazolium iodide (**5**) (Lienhard, 1966) and 2-(1-hydroxyethyl)-3-benzyl-4-methylthiazolium iodide (**6**) (Stivers & Washabaugh, 1992) were prepared as described previously. 3-Methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) and 1-(car-

[4] 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 [Commission on Physical Organic Chemistry, IUPAC (1988) *Pure Appl. Chem. 60*, 1115] and are abbreviated here as $^1H^+$, H; $^2H^+$, D; and $^3H^+$, T.

boxymethyl)pyridinium chloride hydrazide (Girard's reagent P) were purchased from Aldrich. [1,2-^{14}C]Acetaldehyde (Du Pont-NEN, 5.2 Ci/mol), [1-H]acetaldehyde, and [1-D]-acetaldehyde (Aldrich, ≥99 atom % D) were distilled before use. [1-T]Acetaldehyde (0.048 Ci/mol) was prepared from [2-T]nitroethane with the Nef reaction (Leitch, 1955). The specificity for tritium incorporation into the C(1) position of acetaldehyde was demonstrated using ^{1}H NMR: [1-D]-acetaldehyde synthesized from exchange-labeled [1-D]nitroethane contained ≤1% deuterium at the C(2)-methyl position. 3-Hydroxy-2-butanone (acetoin) dimer was vacuum distilled, recrystallized (Berl & Beuding, 1951), and stored at 4 °C in an amber glass bottle under nitrogen. 1-Naphthol was sublimed and stored at room temperature in an amber glass bottle under nitrogen. [3-D]Acetoin was synthesized by zinc-catalyzed reduction of freshly distilled diacetyl in aqueous D_2SO_4 (Diels & Stephan, 1907): mp (white crystalline dimer) 90 °C; ^{1}H NMR (D_2O) δ 1.36 (d, 3 H), 2.22 (s, 3 H); the absence of a quartet at δ 4.42 ppm in the ^{1}H NMR spectrum of the product in D_2O indicates an isotopic purity of ≥99 atom % D. Pyruvamide was synthesized by acid hydrolysis of pyruvonitrile and purified by sublimation (Anker, 1948; Thomas et al., 1951): mp 127 °C (sub); ^{1}H NMR (Me_2-SO-d_6) δ 2.29 (s, 3 H), 7.75 (d, 2 H). Pyruvamide is 60% hydrated based on the integration ratio of the methyl group resonances at δ 2.42 ppm (keto form) and δ 1.54 ppm (hydrate form) in the ^{1}H NMR spectrum in D_2O. PDC holoenzyme was purified from fresh brewers' yeast (Sieber et al., 1983) to a specific activity of 60 units mg^{-1}, where one unit represents the conversion of 1 μmol of pyruvate to acetaldehyde per minute at 30 °C (Ullrich, 1970). The α_4 (α_4-PDC) and $\alpha_2\beta_2$ isozymes were resolved from the PDC isozyme mixture (mix-PDC) using DEAE-Sephadex chromatography as described previously (Kou et al., 1986), except that sodium phosphate buffers were substituted for sodium arsenate buffers.

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. Typical reaction conditions for PDC-catalyzed acetoin formation involved incubation of PDC (1.3–3.5 units mL^{-1}) in a final volume of 250–350 μL at 30 °C in 100 mM sodium citrate buffer (pH 6.00) containing 1 mM $MgSO_4$, 0.12 mM TDP, and 0.010–1.0 M [1-L]acetaldehyde (L = H or D) or [1-T]acetaldehyde (0.047 Ci/mol) in the presence or absence of 100–250 mM pyruvamide. Reactions were initiated by the addition of PDC, which had been preincubated at 30 °C in 100 mM sodium citrate buffer (pH 6.00) containing 5 mM TDP and 1 mM $MgSO_4$ for 15 min.

Determination of Acetoin. [3-L]Acetoin (L = H or D) was determined using the assay described by Westerfeld (Westerfeld, 1945) with minor modifications. Typically, 100-μL aliquots were removed from the enzyme reaction mixture at appropriate time intervals and quenched with 400 μL of 0.05 M HCl. [3-L]Acetoin standards containing 0, 20, and 40 nmol of [3-L]acetoin, each of the reaction components (except PDC), and the corresponding concentrations of acetaldehyde were processed in parallel reactions. Quenched samples (≤9) were placed in a nine-needle Reacti-Vap apparatus (Pierce) in a fume hood. A single 22-gauge needle per sample was immersed 9.5 mm below the surface of the quenched sample solution, and unreacted acetaldehyde was removed (≥95%) by aerating the quenched samples (250 mL min^{-1}) for 60 min; remaining acetaldehyde was shown not to interfere with the acetoin assay. The chromophore was developed for 90 min (L = H) or 3.5 h (L = D). Plots of absorbance at 540 nm against [3-L]acetoin concentration were linear with extinction coefficients of 19 000 ± 500 M^{-1} cm^{-1} (L = H) and 25 300 ± 1100 M^{-1} cm^{-1} (L = D) in the absence or presence of 100 mM pyruvamide.

Scheme II

$$E \underset{k_1}{\overset{k_1[S]}{\rightleftharpoons}} ES \underset{k_2}{\overset{k_2[S]}{\rightleftharpoons}} ES_2 \xrightarrow{k_3} EP \xrightarrow{k_4} E + P$$

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 and [1,2-^{14}C]acetaldehyde specific radioactivity was determined by isolating the 2,4-dinitrophenylhydrazone by HPLC after an extraction step (Fung & Grosjean, 1981). Acetaldehyde was determined by comparing the integrated area of the acetaldehyde 2,4-dinitrophenylhydrazone peak to a standard curve for acetaldehyde 2,4-dinitrophenylhydrazone prepared in the same manner as above. Fractions containing acetaldehyde 2,4-dinitrophenylhydrazone were counted for at least 10^5 counts with automatic quench control.

Data Analysis. Initial rates of acetoin (P, Scheme II) formation, v (M s^{-1} unit^{-1}), were calculated from the linear slopes of plots of [acetoin] against time (3–5 time points), in which the reaction had proceeded ≤2% to completion. Where multiple determinations of initial rates were made, they agreed within ±5% of the average value. Steady-state kinetic parameters were calculated from eq 2, which is the steady-state solution to Scheme II; the terms A (M^2), B (M), and V (M s^{-1}) represent the collection of rate constants given in eqs 3–5.

$$v = \frac{V[S]^2}{A + B[S] + [S]^2} \quad \text{or} \quad \frac{1}{v} = \frac{1}{V}\left(\frac{A}{[S]^2} + \frac{B}{[S]} + 1\right) \tag{2}$$

$$A = \frac{k_{-1}k_4(k_{-2} + k_3)}{k_1k_2(k_3 + k_4)} \tag{3}$$

$$B = \frac{k_2k_3k_4 + k_{-1}k_4(k_{-2} + k_3)}{k_{-1}k_2(k_3 + k_4)} \tag{4}$$

$$V = \frac{[E]_{tot}\, k_3k_4}{k_3 + k_4} \tag{5}$$

Determination of $^T(V/B)$. The competitive steady-state discrimination isotope effect on the rate constant V/B for acetoin formation from [1-T]acetaldehyde catalyzed by α_4-PDC (see eq 2) was determined from the ratio of the specific activities of the acetaldehyde substrate and the solvent product, in which the reaction had proceeded ≤2.5% to completion. The 350-μL enzyme reaction mixture containing 49 mM [1-T]-acetaldehyde was quenched by the addition of 14 μL of 6 M HCl; a 25-μL aliquot was removed for acetoin determination, and a 300-μL aliquot was removed for determination of [^{3}H]-H_2O. The 300-μL quenched sample was aerated to remove acetaldehyde (see above). Remaining carbonyl compounds were converted to nonvolatile hydrazones (Mitchel & Birnboim, 1977) by reacting a 200-μL aliquot of the aerated, quenched sample with 50 μL of 1.0 M Girard's reagent P in 0.5 M sodium formate buffer (pH 2.90) for 15–30 min at 30 °C. [^{3}H]H_2O was sublimed from the derivatized sample, and 50-μL aliquots of the sublimate were analyzed by ion-moderated partition HPLC on a Supelco SUPELCOGEL C-611 column (H^+ form) (7.8 × 300 mm) with isocratic (10

mM H_2SO_4) elution at 70 °C and detection at 210 nm (0.020 AUFS). The experimental retention volume was 9.2 mL for [^{3}H]H_2O, and fractions containing [^{3}H]H_2O were counted for at least 10^5 counts with automatic quench control. [^{3}H]-H_2O specific radioactivity was calculated by dividing the total radioactivity recovered in the [^{3}H]H_2O-containing fractions (after subtraction of background counts) by the amount of acetoin produced after 90 min of reaction. $^T(V/B)$ was calculated with $^T(V/B) = SA^P/SA^S$ (Northrop, 1982), in which SA^P is the specific radioactivity of the solvent product ([^{3}H]-H_2O) and SA^S is the initial specific radioactivity of the acetaldehyde substrate.

Determination of $^{14C}(V/B)$. Discrimination against [1,2-^{14}C]acetaldehyde during catalysis of acetoin formation by α_4-PDC was determined from the ratio of the specific activities of the acetaldehyde substrate and the acetoin product, in which the reaction had proceeded ≤2.5% to completion. α_4-PDC (1.1 units mL^{-1}) was incubated in a final volume of 350 μL at 30 °C in 100 mM sodium citrate buffer (pH 6.00) containing 1 mM $MgSO_4$, 0.34 mM TDP, 50 mM [1,2-^{14}C]acetaldehyde (0.3 Ci/mol), 50 mM sodium pyruvate, and 100 mM pyruvamide. The reaction was quenched by addition of 14 μL of 6 M HCl, the quenched sample was aerated and sublimed to remove acetaldehyde, and acetoin in the sublimate was isolated by reversed-phase (C_{18}) HPLC on a Whatman (EQC 10u I 125A C18) column (4.3 × 216 mm) with isocratic (10 mM $(NH_4)_2SO_4$) elution at ambient temperature and detection at 210 nm (0.010 AUFS). The experimental retention volume was 5.9 mL for acetoin. [3,4-^{14}C]Acetoin specific radioactivity was calculated by dividing the radioactivity (counted for at least 10^5 counts with automatic quench control) in an aliquot of the acetoin-containing fractions (after subtraction of background counts) by the amount of acetoin in an equal aliquot. $^{14C}(V/B)$ was calculated with $^{14C}(V/B) = SA^P/SA^S$, in which SA^P is the specific radioactivity of the product ([3,4-^{14}C]acetoin) and SA^S is the initial specific radioactivity of the [1,2-^{14}C]acetaldehyde substrate.

Statistics. Values are expressed as the mean ± the standard error of the mean. The propagated random error in the calculated value of the Swain–Schaad exponent (y) (eq 1) was calculated as described previously (Washabaugh & Jencks, 1989).

RESULTS

Figure 1 shows the dependence of the steady-state initial velocities for acetoin formation catalyzed by mix- and α_4-PDC on acetaldehyde concentration in the presence and absence of saturating concentrations (100–250 mM) of pyruvamide. The observed initial rates at ≤0.75% conversion to product were linear with no evidence of a lag or "burst" kinetics. Initial velocities for PDC-catalyzed acetoin formation were independent of the method for acetoin determination: identical initial velocities were determined using a gas chromatographic method (Chen & Jordan, 1984) and the modified Westerfeld assay described here. No reverse reaction of acetoin to form free acetaldehyde was detected,[5] which is consistent with previous reports (Singer & Pensky, 1952; Chen & Jordan, 1984).

The following points suggest that the downward curvature at ≥0.6 M acetaldehyde in Figure 1 does not represent PDC inactivation by high concentrations of acetaldehyde and that medium effects attributable to varying [acetaldehyde] are

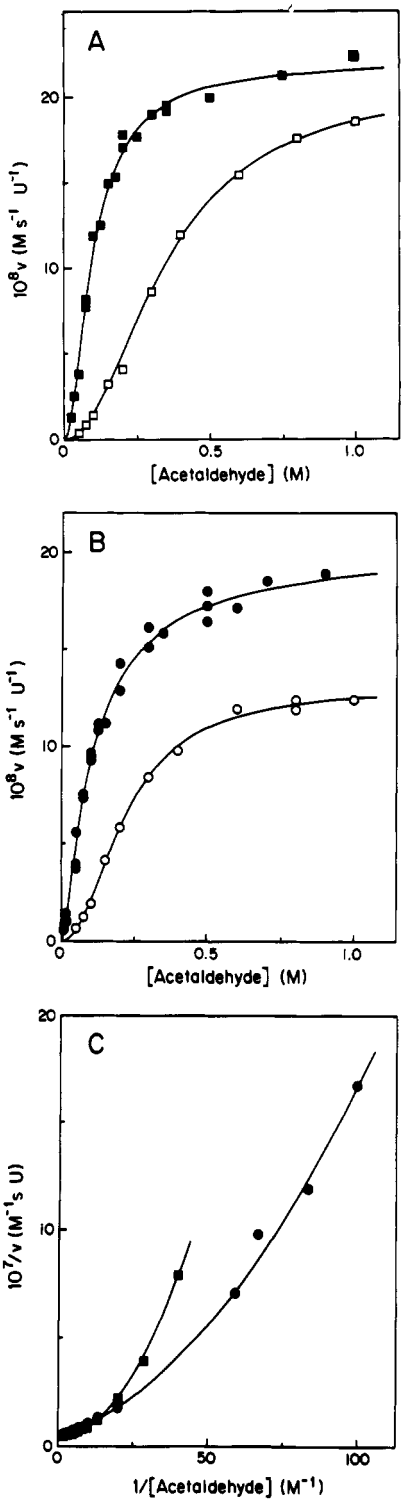


FIGURE 1: (A) Dependence of the initial velocity on the concentration of [1-H]acetaldehyde for acetoin formation catalyzed by mix-PDC in the presence (■) or absence (□) of 100 or 250 mM pyruvamide. (B) Dependence of the initial velocity on the concentration of [1-H]-acetaldehyde for acetoin formation catalyzed by α_4-PDC in the presence (●) or absence (○) of pyruvamide. (C) Double-reciprocal plot for acetoin formation catalyzed by mix-PDC (■) and α_4-PDC (●) in the presence of pyruvamide. PDC (2.5–2.8 units mL^{-1}) was incubated at 30 °C in 100 mM sodium citrate buffer (pH 6.00) containing 1 mM $MgSO_4$ and 0.12 mM TDP. The lines are based on Scheme II, using the rate law given in eq 2 with values for the steady-state kinetic parameters reported in Table I.

[5] Two hundred millimolar acetoin was added to a standard reaction solution containing mix-PDC (2.8 units mL^{-1}) and 100 mM pyruvamide in the presence of excess ADH and NADH; no acetaldehyde (≤1 μM) was detected in a 30-min incubation. This indicates that under identical conditions the reverse reaction is ≥300-fold [=(1.6 × 10^{-7} M s^{-1})/(≤5.5 × 10^{-10} M s^{-1})] less favorable than the reaction to form acetoin.

Table I: Steady-State Kinetic Parameters for Acetoin Formation from [1-L]Acetaldehyde Catalyzed by α_4-PDC and Mix-PDC in the Presence and Absence of Pyruvamide[a]

isozyme	isotope	k_{cat}[b] (s^{-1})	$10^2 A$ (M^2)	$10^2 B$ (M)	k_{cat}/A (M^{-2} s^{-1})	k_{cat}/B (M^{-1} s^{-1})
			Without Pyruvamide			
α_4	H	2.5 ± 0.1	5.1 ± 0.2	≤1.5	49 ± 3	≥170
	D	0.74 ± 0.02	3.7 ± 0.3	≤1.5	20 ± 2	≥50
mix	H	4.0 ± 0.1	13.5 ± 0.8	≤2.6	30 ± 2	≥160
	D	0.83 ± 0.03	3.6 ± 0.5	≤1.2	23 ± 3	≥69
			In the Presence of 100 or 250 mM Pyruvamide			
α_4	H	3.9 ± 0.1	0.35 ± 0.05	8.7 ± 0.8	1120 ± 170	45 ± 5
	D	1.8 ± 0.1	0.64 ± 0.09	6.2 ± 0.6	282 ± 42	29 ± 3
mix	H	4.2 ± 0.1	0.84 ± 0.08	2.1 ± 0.1	504 ± 50	205 ± 115
	D	0.98 ± 0.04	1.0 ± 0.1	≤1	98 ± 14	≥98

[a] L = H or D. At 30 °C in 100 mM sodium citrate buffer (pH 6.00) containing 1 mM $MgSO_4$ and 0.12 mM TDP in the presence of zero or 100–250 mM pyruvamide; the steady-state kinetic parameters are defined in eqs 2–5 (see text). [b] Values of k_{cat} were calculated from V by assuming a maximal specific activity for pure PDC of 80 units mg^{-1} (Sieber et al., 1983), one active site per tetramer, and a molecular mass of 242 kDa for the holoenzyme (Ullrich & Freisler, 1977; Hopmann, 1980; Sieber et al., 1983).

small: (1) The rate of PDC-catalyzed acetoin formation was not significantly affected (≤±5%) by the presence of ≤1.0 M polar organic solvents (ethylene glycol, ethanol, or acetonitrile), which have similar dielectric constants to that of acetaldehyde. A slight increase in the rate of acetoin formation was observed (≤12%) for [organic solvent] = 1.2 M. (2) The value of V (eq 5) for acetoin formation catalyzed by mix-PDC is independent of [pyruvamide] (see Table I; Figure 1A, open squares), although downward curvature occurs at a lower [acetaldehyde] in the presence of pyruvamide (solid squares). (3) Figure 1 provides no evidence that additional terms are required in the rate law (eq 2) to fit the data at high (≥0.5 M) [acetaldehyde]. There is little or no substrate inhibition of acetoin formation by acetaldehyde (K_i > 2 M) because terms for dead-end inhibition representing nonproductive binding of E, ES, ES_2, or EP in Scheme II and eq 2 do not significantly improve the fit to the data. (4) A modest time-dependent loss of enzyme activity was observed in 1.0 M acetaldehyde (≤10% after 15 min), which was circumvented by removing time points at ≤8 min of total reaction time for 0.6–1.0 M acetaldehyde.

Pyruvamide was shown to give saturable activation kinetics with half-maximal activation occurring at 40 mM pyruvamide (data not shown). The steady-state kinetic parameters for acetoin formation were identical within experimental error when 100 or 250 mM concentrations of pyruvamide were used (see Figure 1). We conclude that PDC is saturated with pyruvamide under these conditions and that there is no significant inhibition of the reaction by ≤250 mM pyruvamide. The identical maximal velocities in the presence of 100 or 250 mM pyruvamide exclude the possibility that pyruvamide is contributing to the rate of acetoin formation by providing an additional pathway to product.

A sigmoidal dependence of initial velocity on [substrate] must be interpreted cautiously because sigmoidicity can be attributed to factors unrelated to the reaction mechanism: these include uncertainty in rate measurements at low [substrate] and the presence of compounds in the reaction mixture that combine reversibly or irreversibly with the substrate and render it inactive (Laidler & Bunting, 1973). The following points support the conclusion that sigmoidicity in the presence of pyruvamide (Figure 1) represents the second-order dependence of initial velocity on [acetaldehyde]: (1) There was little or no evaporation of acetaldehyde (≤5%) from the enzyme reaction mixture after 60 min of incubation at 30 °C. (2) A sigmoidal dependence of initial velocity on [acetaldehyde] is observed (Figure 1) by evaluating eq 2 with the maximum and minimum values for initial velocity measurements at ≤100 mM acetaldehyde (≤±20%). (3) No changes in the ultraviolet or ^1H NMR spectrum of pyruvamide that might suggest formation of a stable adduct with acetaldehyde were detected. This is important because amides have been shown to form stable adducts with aldehydes that can be isolated from aqueous solution (Challis & Challis, 1979).

Figure 2 shows the dependence of the steady-state initial velocity on the concentration of [1-D]acetaldehyde (solid circles) for acetoin formation catalyzed by α_4-PDC in the presence and absence of pyruvamide at 30 °C in 100 mM sodium citrate buffer (pH 6.00). The dashed line is the calculated curve for the reaction of [1-H]acetaldehyde under the same conditions. The steady-state kinetic parameters for the reaction of [1-D]acetaldehyde with mix-PDC (data not shown) and α_4-PDC are reported in Table I. Steady-state primary deuterium kinetic isotope effects for acetoin formation, ^DV, $^D(V/A)$, and $^D(V/B)$, are reported in Table II. A previously reported value of ^DV = 4.5 for catalysis of acetoin formation by mix-PDC (Chen & Jordan, 1984) is in reasonable agreement with values of $^DV = 4.2 \pm 0.2$ and 4.9 ± 0.2 reported here for catalysis by mix-PDC in the presence and absence of pyruvamide, respectively (Table II).

Figure 3 shows the dependence of the observed primary deuterium kinetic isotope effect on the concentration of [1-L]-acetaldehyde for acetoin formation catalyzed by α_4-PDC in the presence and absence of pyruvamide in 100 mM sodium citrate buffer (pH 6.00) at 30 °C. Analogous plots for mix-PDC in the presence and absence of pyruvamide are not shown, although the results are qualitatively similar to those seen for α_4-PDC.

The competitive discrimination tritium isotope effect for catalysis by pyruvamide-activated α_4-PDC of acetoin formation from [1-T]acetaldehyde is $^T(V/B) = 2.17 \pm 0.05$ (n = 4). The following control experiments support the conclusion that values of $^T(V/B)$ represent tritium release from the C(α)-position of PDC-bound HETDP: (1) Quantitative recovery of $[^3H]H_2O$ (≥99.5%) was demonstrated with the HPLC procedure outlined under Experimental Procedures. (2) No significant release of tritium (≤0.5% of the total counts recovered in the fractions containing $[^3H]H_2O$ was observed from [3-T]acetoin produced in the reaction[6] or from the adduct

[6] We assume that the specific radioactivity of the product acetoin is equal to the specific radioactivity of the [1-T]acetaldehyde substrate because there would only be a small secondary isotope effect on the addition reaction of acetaldehyde to the C(α)-carbanion/enamine (Palmer & Jencks, 1980).

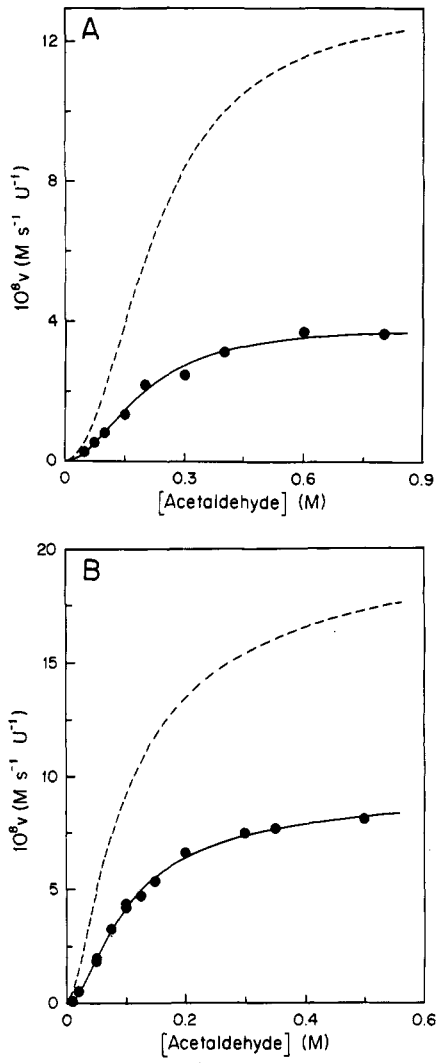


FIGURE 2: Dependence of the initial velocity on the concentration of [1-D]acetaldehyde for acetoin formation catalyzed by α_4-PDC (2.5–5.2 units mL^{-1}) in the absence (A) or presence of 100 mM pyruvamide (B) at 30 °C in 100 mM sodium citrate buffer (pH 6.00) containing 1 mM $MgSO_4$ and 0.12 mM TDP. The solid lines drawn through the [1-D]acetaldehyde data are based on Scheme II, using the rate law given in eq 2 with values for the steady-state kinetic parameters reported in Table I. The dependence of the initial velocity on the concentration of [1-H]acetaldehyde for acetoin formation catalyzed by α_4-PDC under identical conditions is shown for comparison (broken lines).

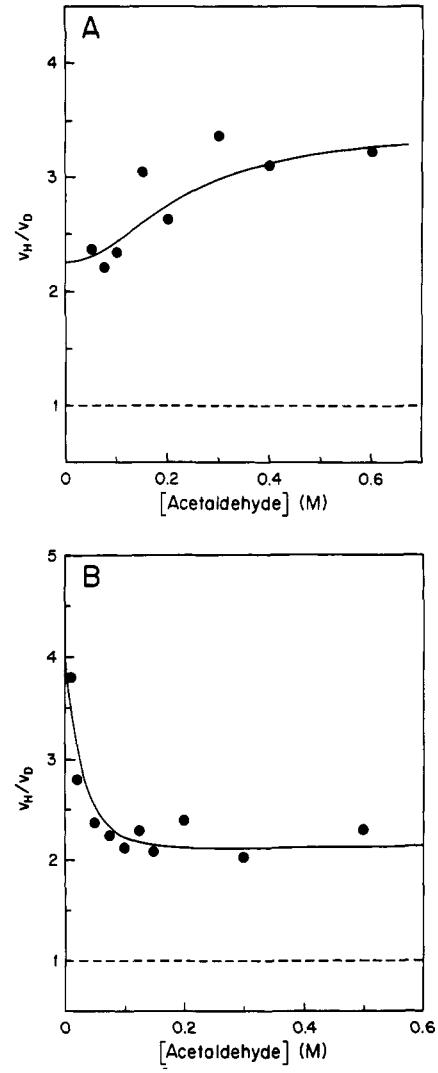


FIGURE 3: Dependence of the observed primary deuterium kinetic isotope effect, v_H/v_D, on the concentration of [1-L]acetaldehyde for catalysis of acetoin formation from [1-L]acetaldehyde by α_4-PDC at 30 °C in 100 mM sodium citrate buffer (pH 6.00) containing 1 mM $MgSO_4$ and 0.12 mM TDP in the absence (A) or presence of 100–250 mM pyruvamide (B). The lines are based on Scheme II, using the rate law given in eq 2 with values for the steady-state kinetic parameters reported in Table I (see text).

Table II: Steady-State Primary Deuterium Kinetic Isotope Effects for Acetoin Formation from [1-L]Acetaldehyde Catalyzed by α_4-PDC and Mix-PDC in the Presence and Absence of Pyruvamide[a]

isozyme	^DV	$^D(V/A)$	$^D(V/B)$
	Without Pyruvamide		
α_4	3.4 ± 0.1	2.5 ± 0.2	3.4[b]
mix	4.9 ± 0.2	1.3 ± 0.2	2.3[b]
	In the Presence of 100 or 250 mM Pyruvamide		
α_4	2.2 ± 0.1	4.0 ± 0.8	1.6 ± 0.2
mix	4.2 ± 0.2	4.7 ± 0.8	2.7[b]

[a] L = H or D. At 30 °C in 100 mM sodium citrate buffer (pH 6.00). The steady-state kinetic parameters are defined in eqs 2–5 (see text). [b] These isotope effects are based on lower limits on V/B for the H and D substrates (see Table I).

of acetoin and Girard's reagent P at 30 °C and pH 2.9. (3) No other radioactive compounds were present in the fractions containing $[^3H]H_2O$. [3-T]Acetoin was resolved from $[^3H]H_2O$ by HPLC, and ≤0.5% total radioactivity in the $[^3H]H_2O$-containing fractions could be attributed to [1-T]acetaldehyde.

The competitive discrimination isotope effect for catalysis by pyruvamide-activated α_4-PDC of acetoin formation from [1,2-^{14}C]acetaldehyde is $^{14C}(V/B)$ = 1.10 ± 0.02 (n = 4).

DISCUSSION

Steady-State Kinetic Mechanism. A general problem when studying an enzyme-catalyzed reaction involving two identical substrate molecules is that the concentration of neither substrate can be varied independently. Therefore, standard methods for determining the order of addition of reactants, such as slope–intercept analysis using double-reciprocal plots (Segal, 1975) and the concentration dependence of kinetic isotope effects (Cook, 1991), are not possible. Despite these limitations, the following conclusions can be made about the kinetic mechanism of this reaction: (1) Previous work (Juni, 1961) demonstrated two acetaldehyde binding sites at the active site of PDC. The first site—the product site in the pyruvate decarboxylation reaction—binds acetaldehyde and noncompetitively inhibits ($K_i \approx 0.1$ M) pyruvate decarbox-

ylation. The second site—the acceptor site for the second molecule of acetaldehyde in the condensation reaction (see Scheme I)—has a K_M value of 10 mM (Chen & Jordan, 1984) for trapping the intermediate produced from saturating concentrations of pyruvate. The intermediate formed from pyruvate decarboxylation—either HETDP or its C(α)-carbanion/enamine—can be trapped by acetaldehyde and the rate of acetoin formation determined (Juni, 1961; Chen & Jordan, 1984). This reaction displays hyperbolic kinetics with respect to [acetaldehyde] with a k_{cat} value of 5.2 s^{-1}: this k_{cat} value was calculated by assuming a maximal specific activity for PDC of 80 units mg^{-1} (Sieber et al., 1983), one active site per tetramer, and a native molecular mass of 242 kDa (Ullrich & Freisler, 1977; Hopmann, 1980; Sieber et al., 1983). (2) The parabolic plot of $1/v$ against $1/$[acetaldehyde] (Figure 1C) indicates the presence of a ternary complex (ESS) at the active site and is inconsistent with a two-step-transfer-type mechanism, which would give a linear double-reciprocal plot (Segal, 1975). Sigmoidal kinetics will always be observed when the concentrations of two substrates are covaried at a constant ratio unless there is an irreversible step separating the reactions of the first and the second substrate (Segal, 1975). (3) A random or sequential order of substrate binding is not revealed by the experiments reported here. However, the observation of two independent acetaldehyde binding sites excludes a simple single site-compulsory ordered mechanism for acetaldehyde binding. (4) These experiments provide no evidence for mechanistically important third-site interactions of acetaldehyde with PDC in the reaction to form acetoin. Previous work indicated that acetaldehyde can interact at a regulatory site on PDC and weakly activate the enzyme for pyruvate decarboxylation (Hübner et al., 1970). This suggests that one additional substrate molecule besides the two that condense to form product may be involved in the reaction to form acetoin. However, these experiments provide no evidence for a third-order term ([acetaldehyde]^3) and can be modeled with a rate law (eq 2) that includes only first- and second-order terms with respect to [acetaldehyde]. (5) Mix-PDC (PDC containing the α_4 and $\alpha_2\beta_2$ isozymes) and α_4-PDC have different steady-state kinetic parameters (Table I) and isotope effects (Table II) for acetoin formation in the presence and absence of pyruvamide: minimally, this means that α_4- and $\alpha_2\beta_2$-PDC are kinetically distinguishable under certain reaction conditions. This finding is important because, kinetically and mechanistically, the two isozymes were reported to be virtually indistinguishable on the basis of experiments involving acetaldehyde formation from pyruvate (Kuo et al., 1986).

We conclude that PDC-catalyzed acetoin formation proceeds by a steady-state mechanism involving a ternary complex between the enzyme and two molecules of acetaldehyde and that there is no significant interaction of acetaldehyde at a third site that affects the steady-state rate of acetoin formation. We have not determined whether substrate addition occurs by a random or a preferred-order mechanism.

Scheme II is a minimal mechanism for acetoin formation, and a random or a preferred order of substrate binding is not implied by this mechanism. The first-order rate constant k_1-[S] represents the net forward rate constant for formation of any ES complex at the active site without consideration for which of the two acetaldehyde binding sites is occupied. If substrate binding occurs by a random mechanism, then k_1[S] would be equal to the sum of the rates of formation of the two possible binary complexes from free substrate and enzyme. Similarly, the rate constant k_2[S] represents the net forward rate constant for the formation of the ternary complex from all pathways. Step three is complex and includes the addition steps as well as the C(α)-proton-transfer step. The rate constant k_3 may contain reversible steps before the first irreversible step, but the minimal mechanism of Scheme II is sufficient for consideration of the kinetic mechanism because the rate constant k_3 is independent of substrate concentration. Similarly, k_4 is the net forward rate constant for product release and may contain reversible steps, which occur before the irreversible release of product. The net forward rate constants k_3 and k_4 are considered irreversible because (1) no acetaldehyde was detected upon incubation of PDC with 0.2 M acetoin at pH 6.00 and 30 °C and (2) PDC binds and forms covalent adducts at the C(2)-position of TDP with a wide range of bulky and nonbulky aldehydes and α-keto acids (Juni, 1961; Lehmann et al., 1973). This suggests that the covalent adduct between acetoin and TDP breaks down to form free acetoin much faster than acetaldehyde release to form enzyme-bound 3.

Nature of the Transition State for the Addition Reaction. Primary kinetic isotope effects on PDC-catalyzed acetoin formation can provide qualitative and quantitative information on the rate-limiting steps in the pathway for conversion of substrate to product, as well as information on the regulatory mechanism of the allosteric effector pyruvamide.

For the reaction in Scheme II the isotope effects on V and V/A are given by eqs 6 and 7, where $^Dk_3$ is the isotope effect on the net forward rate constant for the C(α)-proton-transfer step.

$$^DV = \frac{^Dk_3 + k_3/k_4}{1 + k_3/k_4} \tag{6}$$

$$^D(V/A) = \frac{^Dk_3 + k_3/k_{-2}}{1 + k_3/k_{-2}} \tag{7}$$

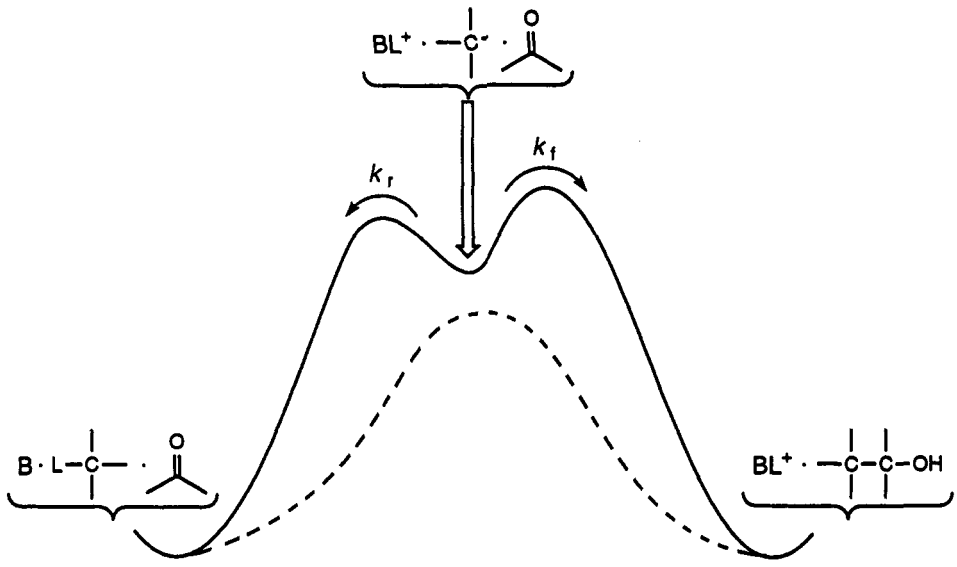
Eqs 6 and 7 show that (1) the observed isotope effect on V will be maximal ($^DV = {}^Dk_3$) when conversion of EP to free enzyme and product is fast compared to the isotopically sensitive step ($k_4 \gg k_3$) and (2) the isotope effect on $^D(V/A)$ will be maximal [$^D(V/A) = {}^Dk_3$] when acetaldehyde is released from the ESS complex much faster than the reaction of ESS to form EP ($k_{-2} \gg k_3$). We conclude that the larger of the isotope effects on V and V/A represents a minimum value for $^Dk_3$. It is important to remember, however, that k_3 is not a microscopic rate constant, and thus, the isotope effect on k_3 ($^Dk_3$) will not be an intrinsic isotope effect (^Dk) unless the first enzyme-bound intermediate following C(α)-proton transfer is irreversibly committed to form product (Cook and Cleland, 1981). The magnitude of the tritium and deuterium isotope effects on V/B for α_4-PDC (see below) strongly suggest that this is the case.

The isotope effect on V/B for the reaction in Scheme II is given by eq 8. In this case, the observed isotope effect is more complex than the $^D(V/A)$ and ^DV effects and does not depend on a single ratio of rate constants.

$$^D(V/B) = \frac{1 + (k_1/k_2)(k_{-2}/^Dk_3 + 1)}{1 + (k_1/k_2)(k_{-2}/k_3 + 1)} \tag{8}$$

For $^D(V/B)$, if either k_{-2} or the ratio k_1/k_2 becomes very large or very small, the isotope effect will approach unity or $^Dk_3$, respectively. Since the rate constant V/B is only significant at intermediate concentrations of substrate, the isotope effects on V/B are not well determined except for the reactions

Scheme III



catalyzed by α_4-PDC in the presence of pyruvamide (see Table II). Therefore, the mechanistic implications of the $^{D}(V/B)$ isotope effects for mix-PDC will not be considered; the $^{D}(V/B)$ isotope effect for pyruvamide-activated α_4-PDC will be considered together with the tritium isotope effect below.

When two transition states are partially rate limiting for base-catalyzed C–L exchange, the amount of internal return (k_r/k_f, Scheme III), or the extent that the transferred hydron L is returned to the carbanion from the protonated base before a step that does not involve hydron transfer, can be estimated from the y value of eq 1 (Streitwieser et al., 1971, 1973). Equation 1 describes the relationship between the primary kinetic isotope effects, $(k_H/k_T)_{obsd}$ and $(k_H/k_D)_{obsd}$; for reactions in which $k_f \approx k_r$ and $k_r^H > k_r^D > k_r^T$, internal return will contribute differently to the two isotope effects. If two or more transition states are rate limiting for addition of the C(α)-carbanion to acetaldehyde (Scheme III, solid line), the isotope effects will fit eq 2 with values of $y < 1.442$ (Washabaugh & Jencks, 1989; Klinman, 1991; Grant & Klinman, 1992). In a concerted-type mechanism (Scheme III, broken line) the isotope effects will fit eq 2 with values of $y = 1.442$ because only one transition state is rate limiting; there is no C(α)-carbanion intermediate, or the step following hydron transfer is relatively fast.

The tritium and deuterium isotope effects on V/B fit the Swain–Schaad equation (eq 1) with $y = 1.7 \pm 0.3$. This value of y is not significantly different from suggested values of y in the range 1.33–1.58 (Stern & Vogel, 1971) and provides evidence against internal return of the transferred hydron L to the C(α)-carbanion (Scheme III, $k_r \approx k_f$). Values of $^{L}(V/B) > 1$ are inconsistent with a stepwise mechanism in which carbon–carbon bond formation is entirely rate limiting (Scheme III, $k_r/k_f \gg 1$). This result provides evidence that catalysis of the addition of HETDP to acetaldehyde by PDC follows either a concerted mechanism (broken line) or a stepwise mechanism in which carbon–carbon bond formation is not rate limiting ($k_r/k_f \ll 1$). The value of $y = 1.7 \pm 0.3$ does not exclude a tunneling component for C(α)-L abstraction ($y > 1.442$) (Klinman, 1991; Grant & Klinman, 1992).

A Swain–Schaad exponent $y \geq 1.442$ *requires* that the observed isotope effect reflect the intrinsic isotope effect—the primary kinetic isotope effect on the proton-transfer step itself—for C(α)-hydron abstraction from PDC-bound HETDP (Northrop, 1982):[7] even moderate internal return markedly depresses observed isotope effects (Washabaugh & Jencks, 1989). The maximum observed V/K-type deuterium isotope effects reported in Table II give a value of $^{D}k = 4.1 \pm 0.4$ for this intrinsic isotope effect.

The value of $^{D}k = 4.1$ for PDC-bound HETDP may be compared with a value of $\approx$9 that was calculated from the differences in zero-point vibrational stretching frequencies of C(α)-H and C(α)-D for related 2-(1-hydroxyethyl)-3-R-4-methylthiazolium ions.[8] The small value of ^{D}k may result from an asymmetrical enzymatic transition state for C(α)-proton transfer between PDC-bound HETDP and a catalytic base [Melander, 1960; Westheimer, 1961; see also Crane and Washabaugh (1993)] or from the incursion of substantial coupling between proton movement and heavy atom motion in the transition state of a concerted mechanism (Engdahl et al., 1983; Murray & Webb, 1991). Model studies have shown that C(α)-proton transfer from HET in aqueous solution has a small kinetic barrier and is always thermodynamically unfavorable in aqueous solution with a pK_a value of $\approx$19, and separation of the proton-transfer products involves significant solvent reorganization (Stivers & Washabaugh, 1992). Further model studies are required to interpret $^{D}k = 4.1$ in terms of enzymatic transition-state structure. Two interesting, and

[7] The overall equilibrium isotope effect, $^{D}K_{eq} = 1.11 \pm 0.03 = 1.13 \pm 0.03/1.016$, for the reaction to form acetoin was estimated from isotope fractionation factors (Cleland, 1980) on the basis of 60% hydrated and 40% unhydrated acetaldehyde [(0.6)(1.14) + (0.4)(0.83) = 1.016] and the fractionation factor for acetoin ($\phi = 1.13 \pm 0.03$), which is not significantly hydrated in aqueous solution (J. T. Stivers and M. W. Washabaugh, unpublished results). This measured value for the fractionation factor of acetoin is substantially larger than $\phi = 1.03 \pm 0.04$ for dihydroxyacetone phosphate, a similar α-hydroxy ketone (Fletcher et al., 1976).

[8] Primary deuterium kinetic isotope effects of $k_H/k_D \approx 9$ at 30 °C for **5** ($pK_a^{C(\alpha)-H} = 22.3$) and **6** ($pK_a^{C(\alpha)-H} = 21.7$) were calculated from the differences in zero-point vibrational stretching frequencies of C(α)-H and C(α)-D by using $k_H/k_D = \exp[h(\upsilon_H - \upsilon_D)/2kT]$; this treatment assumes that the differences in stretching vibrations upon deuteration dominate the measured isotope effect. Although stretching vibrations contribute substantially to the total zero-point energies, contributions from other vibrational modes certainly make a significant contribution to the zero-point energies. The stretching frequencies for C(α)-H of 3220 cm^{-1} for **5** and 3250 cm^{-1} for **6** were measured in KBr pellets as described previously (Washabaugh & Jencks, 1988); C(α)-D stretching frequencies of 2340 cm^{-1} for **5** and 2300 cm^{-1} for **6** were measured similarly.

related, questions regarding the enzymatic transition-state structure for this reaction are (1) the contribution, if any, of desolvation of HETDP at the active site of PDC to catalysis of C(α)-proton abstraction and (2) the contribution of electrophilic catalysis of C(α)-proton abstraction from HETDP by the iminium cation in the thiazolium ring compared to a carbonyl group activated by a proton or a metal ion [see, for example, Gerlt and Gassman (1992)].

A kinetic isotope discrimination of $^{14C}(V/B) = 1.10 \pm 0.02$ against [1,2-^{14}C]acetaldehyde during catalysis of acetoin formation by α_4-PDC, in which the reaction had proceeded $\leq$2.5% to completion, provides evidence against a stepwise mechanism in which carbon–carbon bond formation is not rate limiting ($k_r/k_f \ll 1$). With the absence of significant internal return for C(α)-hydron abstraction and $^{D}k = 4.1 \pm 0.4$, this ^{14}C kinetic isotope also provides evidence that proton movement is coupled to carbon–carbon bond formation in the transition state of a concerted mechanism (Scheme III, broken line). A concerted, coupled mechanism for acetoin formation would be supported by a deuterium kinetic isotope effect on the value for $^{14C}(V/B)$ during the reaction of [$C(\alpha)$-D]HETDP; however, this is a problematic experiment using the methods reported here because of the relatively large (±20%) errors on $^{14C}(V/B)$. The deuterium kinetic isotope effect that arises from a change from [$C(\alpha)$-H]HETDP to [$C(\alpha)$-D]HETDP is expected to result in a small decrease in $^{14C}(V/B)$, but this deuterium kinetic isotope effect is not significantly less than the experimental error in the value.[9]

The mechanism of C(α)-carbanion/enamine addition to carbonyl compounds in aqueous solution has not been investigated in detail. Because the C(α)-carbanion/enamine has a significant lifetime in aqueous solution and is as basic as the similarly "normal" C(2)-ylide derived from thiamin, the aldol-type addition reaction of C(α)-carbanion/enamine must follow either a stepwise pathway or a concerted pathway (Washabaugh & Jencks, 1988). The stepwise pathway must exist, but it appears to be bypassed by a concerted pathway because of the relative instability of, and small barrier for proton transfer to and from (Stivers & Washabaugh, 1992), the carbanion. Concurrent concerted and stepwise pathways are usually only observed for reactions in which one of the steps involves proton transfer between electronegative atoms, which has a small barrier but can cause a large stabilization of the transition state [see, for example, Sørenson and Jencks (1987)]. This has not been observed for similar reactions involving ordinary carbanions in which both processes have larger barriers. However, cleavage of HET to form thiamin and acetaldehyde, a related retrograde aldol-type reaction, proceeds by a nonenforced concerted mechanism in aqueous solution that is determined by the short lifetime of the C(2)-ylide and the small barrier for proton transfer to and from the ylide (Crane & Washabaugh, 1992).

Several workers have shown that there are large intrinsic barriers for addition–elimination reactions involving nitro- and carbonyl-stabilized carbanions that do not involve proton removal; these barriers are much smaller for nitriles (Gilbert, 1980; Bernasconi & Kanavarioti, 1986), which also have smaller intrinsic barriers for proton transfer. We conclude that addition–elimination reactions involving the C(α)-carbanion/enamine that occur during the turnover of TDP-dependent enzymes will most likely have a very small intrinsic barrier.

A value of $k_{non} \approx 10^{-8}$ s^{-1} for nonenzymatic formation of acetoin from acetaldehyde and free thiamin at pH 6.00 and 40 °C in aqueous solution can be estimated using a 1.0 M standard state for thiamin and acetaldehyde.[10] Since $k_{cat} \approx 4$ s^{-1} for the PDC-catalyzed reaction, PDC accelerates this reaction by a factor of $10^{8.6}$-fold = $4/10^{-8}$ compared with 1.0 M reactants in aqueous solution. A fraction of the overall rate enhancement of $10^{8.6}$ may involve stabilization of this moderately unstable carbanion, either to facilitate C(α)-proton abstraction in the transition state of a concerted mechanism or by a factor of $\geq 10^4$ as a discrete intermediate in a stepwise mechanism.[2] For comparison, addition of the C(2)-position of TDP to the keto group of pyruvate is accelerated by a factor of $10^{12.6}$ (Alvarez et al., 1991). The ratio of PDC k_{cat} values in the range 11–20 for pyruvate decarboxylation (45–80 s^{-1}) (Alvarez et al., 1991) and acetoin formation ($\approx$4 s^{-1}), which are dominated by C(2)- ($pK_a = 17.1$)[11] and C(α)-proton abstraction ($pK_a = 18.4$) (Stivers & Washabaugh, 1992), respectively, is nearly identical to the ratio of the equilibrium constants for proton abstraction from these "normal" carbon acids in aqueous solution. This suggests nearly identical values of ΔpK_a ($= pK_a^{BH} - pK_a^{C(\alpha)\text{-}H}$) for proton transfer between a catalytic base on PDC and the two PDC-bound carbon acids because proton removal from normal acids occurs at the maximum possible rate for a given equilibrium constant.

A discrete PDC-bound C(α)-carbanion/enamine intermediate was inferred from the observation of PDC-catalyzed HETDP oxidation by molecular oxygen (Abell & Schloss, 1991). However, in general it is not possible to determine whether a trapping reaction proceeds by a stepwise or a concerted mechanism on the basis of an observed rate. Moreover, the interpretation of experiments designed to trap the putative PDC-bound C(α)-carbanion/enamine with molecular oxygen is complicated because expression of oxygenase activity depends on the ability of PDC to stabilize the initially formed peroxide anion (Abell & Schloss, 1991). Existence of the C(α)-carbanion/enamine as a discrete enzymatic intermediate on TDP-dependent enzymes was also inferred, in part, from a UV spectrum consistent with an extended enamine after reaction of PDC with (*E*)-2-oxo-4-(4-R-phenyl)-3-butenoic acids that are mechanism-based inhibitors (Zeng et al., 1991) and from ^{13}C and solvent deuterium isotope effects and partitioning between inhibition and normal substrate turnover of [*p*-(halomethyl)benzoyl]formates by benzoylformate decarboxylase (EC 4.1.1.7) (Dirmaier et al., 1986; Reynolds et al., 1988; Weiss et al., 1988).

Mechanism of Pyruvamide Activation. Scheme IV is a minimal mechanism for the reaction of PDC with acetaldehyde in the presence of pyruvamide (A). The rate constants k_1' and k_{-1}' correspond to the activation steps, which have been

[9] The deuterium isotope effect on $^{14C}(V/B)$ was estimated using the following equation with intrinsic isotope effects of $^{14}k = 1.10 \pm 0.02$ and $^{D}k = 4.1 \pm 0.4$ and "commitments" of $C_r = 0$ and $C_f = 0$–3 (Cleland, 1991):

$$^{14C}(V/B)_D = \frac{^{14}k + C_f/^{D}k + C_r{}^{14}K_{eq}{}^{D}K_{eq}/^{D}k}{1 + C_f/^{D}k + C_r{}^{D}K_{eq}/^{D}k}$$

A value of $C_f = 3$ results in a small decrease in the competitive discrimination isotope effect against [1,2-^{14}C]acetaldehyde; $^{14C}(V/B)_D = 1.06 \pm 0.03$.

[10] The value of k_{non} was calculated from $k_{non} = k[HO^-][\text{acetaldehyde}]^2$ where $k \approx 1$ M^{-3} s^{-1} at 40 °C in aqueous solution (Yatco-Manzo et al., 1959), $[HO^-] = 10^{-8}$ M at pH 6.00, and [acetaldehyde] = 1.0 M. We assume [thiamin] = 1.0 M.

[11] This pK_a for C(2)-H on thiamin in H_2O was calculated from $pK_a = 17.7$ in D_2O (Washabaugh & Jencks, 1988) by subtracting $\Delta pK_a \approx 0.6$ for the solvent deuterium isotope effect for thiamin (Schowen & Schowen, 1982).

Scheme IV

$$E + A \underset{}{\overset{K_1'}{\rightleftharpoons}} AE \underset{k_1'}{\overset{k_1'}{\rightleftharpoons}} A^*E \underset{k_1}{\overset{k_1[S]}{\rightleftharpoons}} A^*ES \underset{k_2}{\overset{k_2[S]}{\rightleftharpoons}} A^*ES_2 \xrightarrow{k_3} A^*EP \xrightarrow{k_4} A^*E + P$$

studied in detail for pyruvate decarboxylation. Rapid equilibrium binding of pyruvamide (A) to the regulatory site occurs with a dissociation constant $K_1' = 8$ mM followed by a slow, reversible isomerization step ($k_1' = 0.6\ s^{-1}$ and $k_{-1}' = 0.04\ s^{-1}$) to form the activated enzyme complex (A*E) (Hübner et al., 1978). The steps that follow formation of A*E are analogous to the steps shown in Scheme II for the unactivated enzyme. In the presence of saturating pyruvamide all the enzyme is in the A*E form, making steps involving E and AE kinetically transparent; consequently, these steps will be excluded from the following steady-state kinetic analysis. Substitution of $[A^*E]_{tot}$ for $[E]_{tot}$ in the expression for V (eq 5) gives the steady-state expression for the reactions in the presence of saturating concentrations of pyruvamide (Scheme IV). Allosteric activation was suggested to involve a decrease in a kinetic barrier for a step occurring after carbon dioxide release (Gish et al., 1988) and, more specifically, decreases in the kinetic barriers for formation and decay of the putative C(α)-carbanion/enamine intermediate derived from HETDP (Zeng et al., 1991).

Free energies of activation and acetaldehyde binding at pH 6.00 and 30 °C (Figure 4)[12] provide evidence that pyruvamide-activated α_4-PDC has a greater affinity than unactivated α_4-PDC for acetaldehyde occupying the low-affinity "product" site: the observed ≤8-fold rate enhancement in the presence of pyruvamide occurs through an increase in [ES]. This suggests that acetaldehyde release from activated α_4-PDC is *thermodynamically* less favorable in the pyruvate decarboxylation reaction, but does not exclude the possibility that pyruvamide activation decreases the *rate* of acetaldehyde release, compared to unactivated α_4-PDC.

The magnitudes of the $^D(V/A)$ and ^DV isotope effects in the presence and absence of pyruvamide provide additional evidence as to which steps in the reaction pathway are affected by pyruvamide activation. Values of $k_3/k_4 = 2.0 \pm 0.2$ and $k_3/k_{-2} \leq 0.2$ for pyruvamide-activated α_4-PDC were calculated using eqs 6 and 7 with a mean value of $^Dk_3 = 4.5$ (Table II): the maximum isotope effects in Table II for α_4-PDC and mix-PDC are not significantly different, which supports the assumption that $^Dk_3$ is the same for both isozymes. Values of $k_3/k_4 = 0.5 \pm 0.2$ and $k_3/k_{-2} = 1.4 \pm 0.2$ were calculated for unactivated α_4-PDC. These ratios indicate that besides stabilizing the ES and ESS complexes (see Figure 4), pyruvamide decreases the partitioning ratio k_3/k_{-2} by at least 7-fold on α_4-PDC. The ≥7-fold decrease in k_3/k_{-2} suggests that pyruvamide increases the rate constant for release (k_{-2}) of one or both substrates from the ternary complex on α_4-PDC because pyruvamide gives only a ≤4-fold increase in k_3/k_4. This conclusion is supported by the data in Figure 3B, which clearly shows that, in the presence of pyruvamide, the observed isotope effect increases as the rate becomes dominated by the V/A term—at low [S]—whereas the opposite effect is observed in Figure 3A without pyruvamide.

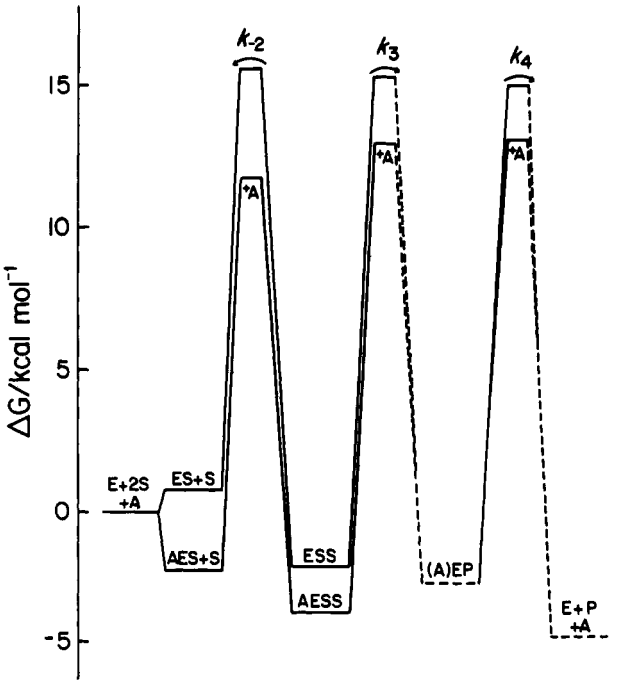


FIGURE 4: Standard-state free energy diagram for catalysis of acetoin formation by α_4-PDC. This diagram is based on Scheme II, using eqs 2–5 with the rate constants $k_{-2} \geq 60\ s^{-1}$, $k_3 = 12\ s^{-1}$, and $k_4 = 6\ s^{-1}$ for pyruvamide-activated α_4-PDC (A) and the rate constants $k_{-2} = 2.8\ s^{-1}$, $k_3 = 4\ s^{-1}$, and $k_4 = 8\ s^{-1}$ for unactivated α_4-PDC (see text).[12] We assume a standard state of 1.0 M for enzyme and substrate. The diagram illustrates changes in the partitioning of the acetaldehyde/α_4-PDC ternary complex (k_3/k_{-2}), and changes in the kinetic barriers corresponding to k_3 and k_4, in the presence and absence of the allosteric activator pyruvamide.

Pyruvamide has no effect on k_{cat} for mix-PDC and confers only a modest 1.6-fold increase in k_{cat} for α_4-PDC. These effects on k_{cat} provide no evidence for a large (≈50-fold) (Zeng et al., 1991) pyruvamide-induced increase in the rate of formation or decay of the putative C(α)-carbanion/enamine in the reaction to form *acetoin*—a large pyruvamide-induced increase in the rate constant k_3 or k_4. An increase in the rate constant k_4 would result in a larger isotope effect on V (see eq 6) compared to the unactivated enzyme, which is opposite to the observed decrease in ^DV. We conclude that the observed 1.6-fold rate enhancement for α_4-PDC under V conditions in the presence of pyruvamide is consistent with a small decrease in a kinetic barrier within the isotopically sensitive step (k_3) and not the product-release step (k_4).

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[12] This profile was constructed using $\Delta G^* = -RT \ln[k(h/k_BT)]$ with the rate constants $k_{-2} \geq 60\ s^{-1}$, $k_3 = 12\ s^{-1}$, and $k_4 = 6\ s^{-1}$ for pyruvamide-activated α_4-PDC (Scheme IV) and the rate constants $k_{-2} = 2.8\ s^{-1}$, $k_3 = 4\ s^{-1}$, and $k_4 = 8\ s^{-1}$ for unactivated α_4-PDC (Scheme II). The rate constants k_3 and k_4 were calculated using eq 6 with the values of k_{cat} in Table I for α_4-PDC and $^Dk_3 = 4.5$; the rate constant k_{-2} was calculated using the ratio k_3/k_{-2}. Activation free energies ΔG^* (ESS → ESS*), ΔG_1^* (ES + S → ESS*), and ΔG_2^* (E + 2S → ESS*), in the presence and absence of pyruvamide, were calculated with the rate constants k_{cat}, k_{cat}/B, and k_{cat}/A reported in Table I, assuming a standard state of 1.0 M for enzyme and substrate. The apparent free energies of acetaldehyde binding were calculated from the activation free energies using ΔG_1 (ES + S → ESS) = $\Delta G^* - \Delta G_1^*$, ΔG_2 (E + 2S → ESS) = $\Delta G^* - \Delta G_2^*$, and ΔG_{12} (E + 2S → ES + S) = $\Delta G_2 - \Delta G_1$.

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