Mechanism of Reconstitution of Brewers' Yeast Pyruvate Decarboxylase with Thiamin Diphosphate and Magnesium†

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ABSTRACT: Reconstitution of apo-pyruvate decarboxylase isozymes (PDC, EC 4.1.1.1) from *Saccharomyces carlsbergensis* was investigated by determination of the steady-state kinetics of the reaction with thiamin diphosphate (TDP) and Mg^{2+} in the presence and absence of substrate (pyruvate) or allosteric effector (pyruvamide). Reconstitution of the PDC isozyme mixture and α_4 isozyme (α_4-PDC) exhibits biphasic kinetics with 52 ± 11% of the PDC reacting with $k_1 = (1.0 \pm 0.3) \times 10^{-2}$ s^{-1} and 48 ± 12% of the PDC reacting with $k_2 = (1.1 \pm 0.6) \times 10^{-1}$ s^{-1} when TDP ($K_{TDP} = 0.5 \pm 0.2$ mM) is added to apo-PDC equilibrated with saturating Mg^{2+}. PDC reconstitution exhibits first-order kinetics with $k_1 = (1.6 \pm 0.5) \times 10^{-2}$ s^{-1} upon addition of Mg^{2+} ($K_{Mg^{2+}} = 0.2 \pm 0.1$ mM) to apo-PDC equilibrated with saturating TDP. Biphasic kinetics for the PDC isozymes provides evidence that apo-PDC reconstitution with TDP and Mg^{2+} involves two pathways, TDP binding followed by Mg^{2+} (k_1) or Mg^{2+} binding followed by TDP (k_2). This is supported by a change in reconstitution pathway with the order of cofactor addition and is inconsistent with a single pathway involving ordered binding of the metal ion followed by TDP. The presence of pyruvamide has no significant effect on the rate constants for apo-PDC reconstitution and favors the k_2 pathway; pyruvate decreases the value of k_2 ≤3-fold and has no effect on the value of k_1. These results are summarized in a model for apo-PDC reconstitution, and implications for mechanistic studies on PDC are discussed.

Pyruvate decarboxylase (PDC)[1] (2-oxoacid carboxylyase, EC 4.1.1.1) catalyzes the nonoxidative decarboxylation of pyruvate to form acetaldehyde [see pp 307−312 of Kluger (1992)] and an aldol-type condensation reaction between two molecules of acetaldehyde to form the α-ketol acetoin (Chen & Jordan 1984; Stivers & Washabaugh, 1993). The tetrameric PDC holoenzyme (holo-PDC) from brewers' yeast (*Saccharomyces carlsbergensis*) contains one thiamin diphosphate (TDP) and one divalent cation (Mg^{2+})[2] per subunit (Dyda et al., 1993). There are at least two holo-PDC isozymes (Kuo et al., 1986; Farrenkopf & Jordan, 1992),[3] designated α_4 (α_4-PDC) and $\alpha'_2\beta_2$, which are kinetically and mechanistically distinguishable on the basis of experiments involving acetoin formation from acetaldehyde (Stivers & Washabaugh, 1993).

Steady-state mechanistic studies on *S. carlsbergensis* PDC have contributed greatly to our understanding of catalysis by TDP-dependent enzymes [see, for example, Alvarez et al. (1991)]. Single-turnover studies should further improve our understanding by allowing for direct observation of events occurring at the holo-PDC active site [see, for example, Crane et al. (1993)]. Optimal sensitivity for product detection and isotope effect measurement in certain steady-state and single-turnover experiments [see pp 19−36 of Johnson (1992)] with holo-PDC-bound coenzyme (TDP or an analog) requires rapid and efficient reconstitution of apo-PDC with coenzyme (TDP or an analog) and Mg^{2+}. Knowledge of the mechanism of PDC reconstitution with TDP and Mg^{2+} is also required before catalysis by PDC-bound coenzyme analogs [see, for example, Golbik et al. (1991)] can be evaluated.

We report here a study of the mechanism of *S. carlsbergensis* apo-PDC reconstitution with TDP and Mg^{2+} in the presence and absence of substrate (pyruvate) and nonsubstrate allosteric effector (pyruvamide). We confirm and extend the observation (Nafe et al., 1972; Golbik et al., 1991)

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[1] Abbreviations: PDC, pyruvate decarboxylase; TDP, thiamin diphosphate; Me_2SO, dimethyl sulfoxide; ADH, alcohol dehydrogenase; NADH (NAD^+), reduced (oxidized) nicotinamide adenine dinucleotide; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonate; EDTA, (ethylenedinitrilo)tetraacetate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonate); ADP, adenosine 5′-diphosphate; U, enzyme activity unit.

[2] The value of V_{max} is independent of the divalent cation, but the stability of the holoenzyme is sensitive to the nature of the divalent cation (Schellenberger, 1967; Schellenberger & Hübner, 1967). The divalent cation affects the reconstitution rate in the order $Ca^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$ (Schellenberger et al., 1966) which follows the order expected if rates of complexation with most ligands are important (Eigen & Hammes, 1963). Relative ratios of the rates of binding of TDP to apo-PDC in the presence of Mn^{2+} (4), Mg^{2+} (1), and Ca^{2+} (0.2) were suggested to provide evidence against the importance of ligand complexation rates (Sanemori et al., 1974). However, facile dissociation of Ca^{2+} from $(Ca^{2+})_4$-holoenzyme under the assay conditions was not ruled out.

[3] M. W. Washabaugh, J. A. Vaccaro, and T. K. Harris, unpublished results.

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that complete reconstitution of apo-PDC occurs significantly faster than widely believed on the basis of preliminary kinetic studies (Schellenberger, 1967; Schellenberger & Hübner, 1967). We conclude that (1) single-turnover and related experiments on pyruvamide-activated PDC that require relatively rapid reconstitution of the apoenzyme are feasible; (2) apo-PDC reconstitution with TDP and Mg^{2+} involves two different pathways which depend on the order of cofactor addition, TDP binding followed by Mg^{2+} or Mg^{2+} binding followed by TDP; (3) apo-PDC isozymes are not kinetically distinguishable on the basis of reconstitution with TDP and Mg^{2+}; (4) pyruvamide has no significant effect on the rate constants for PDC reconstitution; and (5) pyruvate decreases the rate constant ≤3-fold for one reconstitution pathway. We suggest a model for apo-PDC reconstitution and discuss implications for mechanistic studies on pyruvamide-activated PDC isozymes.

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). Stock solutions containing 100 mM TDP hydrochloride were adjusted to pH 6.00 by dropwise addition of 1.0 M NaOH with vigorous magnetic stirring.[4] Sodium 2-(*N*-morpholino)ethanesulfonate (MES) and TDP were purified from contaminating divalent cations by passage of a pH 6.00 aqueous solution through Chelex-100 (Na^+) (Bio-Rad) ($[Mg^{2+}] \leq 30$ nM). Pyruvamide was synthesized by acid hydrolysis of pyruvonitrile and purified by sublimation (Anker, 1948; Thomas et al., 1951): mp 127 °C (sub); ^1H NMR (Me_2SO-d_6) δ 2.29 (s, 3H), 7.75 (d, 2H). Pyruvamide is 60% hydrated on the basis of the integration ratio of the methyl group resonances at $\delta = 2.42$ ppm (keto form) and $\delta = 1.54$ ppm (hydrate form) in the ^1H NMR spectrum in D_2O (Stivers & Washabaugh, 1993). PDC holoenzyme (holo-PDC) was purified from fresh brewers' yeast (Sieber et al., 1983) to a specific activity of 52−60 U mg^{-1}, where one unit (U) 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 mix-PDC using DEAE-Sephadex chromatography as described previously (Stivers & Washabaugh, 1993). The PDC apoenzyme (apo-PDC) was prepared as described previously (Ullrich, 1970; Gubler & Wittorf, 1970); residual divalent cations were removed ($[Mg^{2+}] \leq 30$ nM) by dialysis against three changes of 500 mL of 100 mM Tris HCl (pH 8.5) buffer containing 1 mM EDTA and 3 mL of Chelex-100 (Na^+) (in a separate dialysis bag) for ≥2 h per change.

General Methods. Protein was determined with bicinchoninic acid (Smith et al., 1985) with a bovine serum albumin standard. Dialysis was performed at 4 °C with magnetic stirring in Spectra/Por 2 dialysis tubing (Spectrum) that had been treated and stored as described previously (Washabaugh & Collins, 1984). Typical reaction conditions for apo-PDC reconstitution involved incubation of apo-PDC (0.06−23 mg mL^{-1}, 0.26−95 μM) in a final volume of 200 μL at 30.0 ± 0.2 °C in 75−100 mM sodium MES (pH 6.00) buffer containing 0−10 mM $MgSO_4$, 0−100 mM TDP, 0−100 mM pyruvamide, and 0−33 mM sodium pyruvate. The reaction solution was removed from the constant temperature bath for about 5 s every 0−20 min in order to obtain a 5−20 μL sample that was immediately mixed with 1−3 mL of a solution containing 100 mM sodium MES (pH 6.00) buffer, 0−10 mM $MgSO_4$, 0−10 mM TDP, 171 μM NADH, and 22 U of yeast alcohol dehydrogenase (ADH; alcohol:NAD^+ oxidoreductase; EC 1.1.1.1). The fraction of apo-PDC reconstituted to form active holo-PDC was determined by the following of the decrease in absorbance at 340 nm due to the oxidation of NADH to NAD^+ upon the addition of sodium pyruvate to a final concentration of 33 mM (Ullrich, 1970). Plots of absorbance at 340 nm against time were linear, except for the characteristic lag due to the activation of holo-PDC at very early time points in the absence of pyruvamide (Schellenberger, 1967; Schellenberger & Hübner, 1967; Ullrich, 1970). The absence of downward curvature in these plots supports the conclusion that reconstitution of PDC was quenched by dilution into assay buffer and no further reconstitution occurred in the presence of substrate under these conditions.

Rapid-quench experiments with mix-PDC were performed with a KinTek Instruments Model RQF-3 rapid-quench-flow apparatus operating in "four-syringe" mode and thermostatted at 30.0 ± 0.2 °C. "Reaction buffer" refers to 100 mM sodium MES (pH 6.00) buffer in H_2O containing 10 mM $MgSO_4$ and 100 mM pyruvamide unless otherwise stated. One sample loop was loaded with 35 μL of 75 μM (18 mg mL^{-1}) mix apo-PDC in 300 mM sodium MES (pH 6.64) buffer containing 30 mM $MgSO_4$ and 300 mM pyruvamide. The other sample loop contained 35 μL of 20 mM TDP in 50 mM HCl. The left and right drive syringes were filled with reaction buffer, and the middle drive syringe was filled with H_2O. The reconstitution reaction was initiated by the mixing of the contents of the sample loops to obtain a solution containing 37.5 μM mix apo-PDC, 150 mM sodium MES (pH 6.00) buffer, 15 mM $MgSO_4$, 150 mM pyruvamide, and 10 mM TDP that was allowed to react for a time t_1 ($0 \leq t_1 \leq 180$ s) before the reaction solution (255 μL) was quenched by dilution into 6.4 mL of reaction buffer. Mix holo-PDC produced at each time point (t_1) was determined as described above.

Kinetic modeling was performed using KINSIM, a kinetic modeling program (Barshop et al. 1983).

Electrophoretic Studies. Analytical polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed on 9% polyacrylamide gels according to the method of Laemmli (1970). Samples (4 μg) were denatured by being heated at 100 °C for 2 min in the buffer described by Laemmli (1970). Slab gels (0.15 × 16 × 18 cm, 37-mL bed volume) were developed at 80 V (constant) for 16 h or until the tracking dye reached the bottom of the slab. Gels were stained with Coomassie Brilliant Blue R-250 and destained as described by Wilson (1983). The proteins used as molecular weight standards were rabbit muscle phosphorylase *b* (10^{-3} $M_r = 97$), bovine serum albumin (10^{-3} $M_r = 68$), hen egg white ovalbumin (10^{-3} $M_r = 45$), bovine carbonic anhydrase (10^{-3} $M_r = 30$), and soybean trypsin

[4] There was no detectable hydrolysis of TDP ($R_f = 0.13$) to form thiamin ($R_f = 0.35$) or thiamin monophosphate ($R_f = 0.25$) as determined by analysis of 0.5 μL aliquots of the 100 mM TDP solution (pH 6.00) by thin-layer chromatography [silica gel (200 μm) in an inert binder containing a fluorescent indicator] in ethanol:water (60:40) (v/v) or reversed phase (C_{18}) HPLC (Hollenbach, 1995).

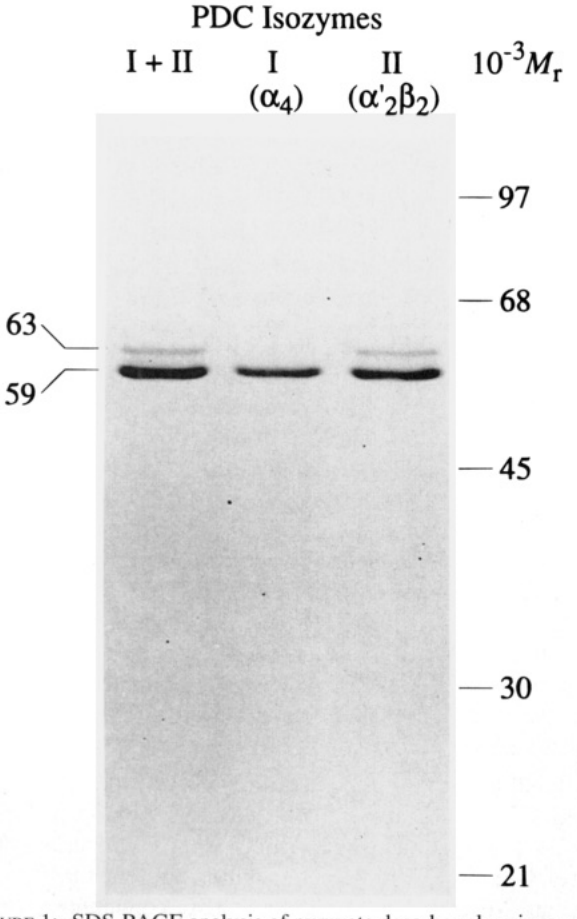


FIGURE 1: SDS-PAGE analysis of pyruvate decarboxylase isozymes (PDC; EC 4.1.1.1) from *S. carlsbergensis*. A 9% polyacrylamide gel was developed in SDS according to the method of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. The lane labeled PDC I + II contained 4 μg of mix-PDC, and the lanes labeled PDC I and PDC II contained 4 μg each of the resolved α_4 (PDC I) and $\alpha'_2\beta_2$ (PDC II) isozymes. The specific activities of mix-PDC and the resolved isozymes were 60 and 52 U mg^{-1}, respectively. Mobility was determined relative to the migration of the dye front. The relative mobilities of the α (or α') and β subunits of PDC correspond to 10^{-3} M_r values of 59 and 63, respectively. Densitometric analysis of the mix-PDC gave an α-(α'):β subunit ratio of 4.2:1 (see text).

inhibitor (10^{-3} M_r = 21) (Bio-Rad).[5] Lanes containing mix-PDC and α_4-PDC were scanned with an AMBIS Radioanalytic Imaging System and analyzed with Quantprobe software (Ver. 4.31).

RESULTS

SDS-PAGE analysis of *S. carlsbergensis* unresolved PDC isozymes (mix-PDC) and the resolved isozymes (α_4-PDC and $\alpha'_2\beta_2$-PDC) is shown in Figure 1; 4 μg of heavily stained protein from the last step of the purification procedure (Sieber et al., 1983) gives mix-PDC with apparent subunit molecular weights of 10^{-3} M_r = 59 ± 2 (α or α') and 63 ± 2 (β). Densitometric analysis of the lanes containing mix- and α_4-PDC gave an $\alpha(\alpha')$:β subunit ratio of 4.2:1 for the mix-PDC isolated from fresh brewers' yeast, which establishes (1) the purity (≥95%) of the mix- and α_4-PDC and (2) the $\alpha(\alpha')$:β subunit ratio in the mix-PDC samples for the experiments reported here. This is important because α_4- and $\alpha'_2\beta_2$-PDC are kinetically and mechanistically distinguishable under certain reaction conditions (Stivers & Washabaugh, 1993) and $\alpha(\alpha')$:β subunit ratios in the range 2−13:1 were obtained in mix-PDC isolated from different sources of brewers' yeast.[6]

The apo-PDC preparations contained ≤0.008 mol of TDP per mole of tetrameric PDC based on a spectrofluorometric assay for the thiochrome derivative of TDP (Airth & Foerster, 1970), had a specific activity of ≤0.5 U mg^{-1} for pyruvate decarboxylation under the above reconstitution conditions without TDP and Mg^{2+} or in the presence of Mg^{2+} without TDP, and had a specific activity of ≤0.5 U mg^{-1} (Chelex-100-treated apo-PDC, see above) or ≤18 U mg^{-1} (untreated apo-PDC) for pyruvate decarboxylation in the presence of TDP without Mg^{2+}. Minimally, these results support the conclusion that it is difficult [see, for example, Lautens and Kluger (1992)], but not impossible, to prepare apo-PDC that is quantitatively free of divalent cations.

Figure 2 shows that reconstitution of apo-PDC·Mg^{2+} (apo-PDC preincubated with saturating Mg^{2+}) with saturating TDP exhibits biphasic kinetics in the presence (solid circles) and absence (open circles) of pyruvamide. Formation of holo-PDC involves an initial "burst" [k^2_{obsd} = (1.1 ± 0.6) × 10^{-1} s^{-1}], which represents 48 ± 12% of the total reconstituted PDC activity. Biphasic kinetics were observed whether the apo-PDC was derived from mix- (panel A) or α_4-PDC (panel B) holoenzyme. Under the reaction conditions of ≥1.6 × 10^4:1 TDP:apo-PDC·Mg^{2+}, the magnitudes of the burst and the observed rate constant for reconstitution in the slow phase [k^1_{obsd} = (1.0 ± 0.3) × 10^{-2} s^{-1}] are independent of PDC isozyme identity and pyruvamide concentration in the range 0−100 mM. The magnitude of the burst is also independent of [PDC] in the range 0.21−37.5 μM (see Figures 2 and 3). These results confirm and extend a previous report that reconstitution of apo-PDC·Mg^{2+} with TDP exhibits zero-order kinetics in apo-PDC at ≥10^4:1 TDP:apo-PDC (Nafe et al., 1972). The burst is not due to contamination of apo-PDC with holo-PDC or residual TDP because apo-PDC retained <1% PDC activity and contained no detectable TDP (see above). Preincubation of the apo-PDC with saturating Mg^{2+} excludes the possibility that the burst is due to residual Mg^{2+}. Where multiple determinations of k^1_{obsd} and k^2_{obsd} were made, they typically agreed within ≤±9% and ≤±18% of the average value, respectively.

Reconstitution of mix apo-PDC·Mg^{2+} (0.2 μM, 0.04 mg mL^{-1}) with 10 mM TDP (5 × 10^4:1 TDP:apo-PDC·Mg^{2+}) in the presence of saturating (≥30 mM) pyruvate exhibits simple first-order kinetics with k^2_{obsd} = (3.3 ± 0.6) × 10^{-2} s^{-1}. Reconstitution of mix apo-PDC·TDP (0.3 μM, 0.06 mg mL^{-1}) with 10 mM $MgSO_4$ (3 × 10^4:1 Mg^{2+}:apo-PDC·-TDP) in the presence of saturating (≥30 mM) pyruvate exhibits simple first-order kinetics with k^1_{obsd} = (1.3 ± 0.5) × 10^{-2} s^{-1} (data not shown).

[5] R. McMacken, unpublished results.

[6] The α and α' subunits are distinguishable by anion-exchange HPLC on a Pharmacia Mono Q HR5/5 column (5 × 50 mm) [equilibrated with 10 mM sodium PIPES (pH 6.3) buffer containing 10 μM TDP, 1 mM $MgSO_4$, and 3 mM 2-mercaptoethanol (load buffer)] with linear gradient elution (0.6 mL min^{-1}) at 4 °C and detection at 280 nm. The 0−100% linear gradient in load buffer containing 150 mM KCl was developed for 90 min. The experimental retention volume was 20 mL for α and 34−37 mL for α' (J. A. Vaccaro and M. W. Washabaugh, unpublished results). Experiments to characterize the structural differences between PDC subunits are in progress.

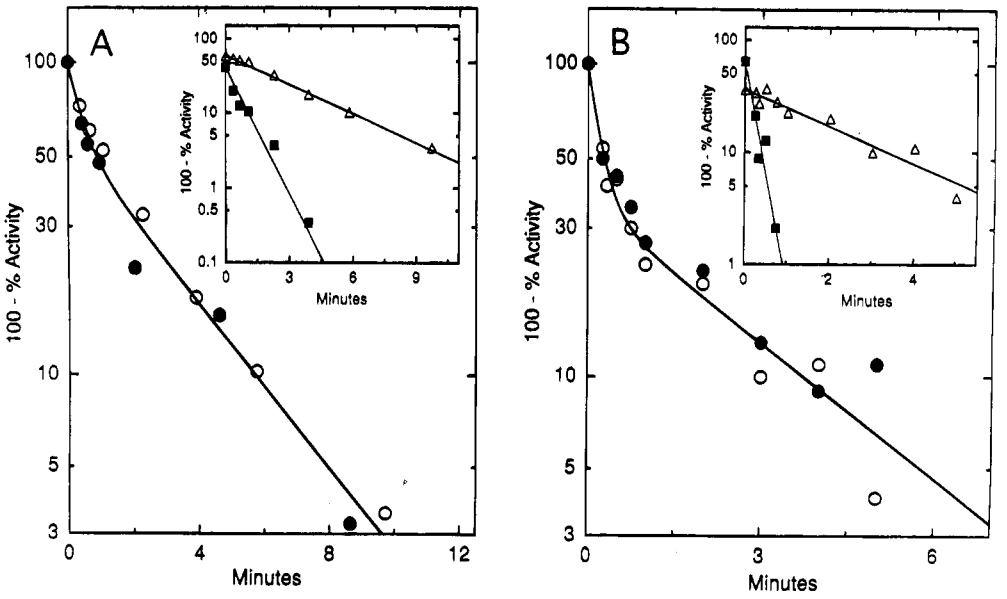


FIGURE 2: (A) Reconstitution of mix-PDC·Mg^{2+} apoenzyme (1.5 mg mL^{-1}, 6.25 μM) with 100 mM TDP in the presence (●) or absence (○) of 100 mM pyruvamide in 75 mM sodium MES (pH 6.00) buffer containing 7.5 mM $MgSO_4$ at 30 °C. The line is drawn for reaction of 41 ± 13% of the PDC with k^2_{obsd} = (3.8 ± 1.6) × 10^{-2} s^{-1} and 59 ± 12% of the PDC with k^1_{obsd} = (5.2 ± 1.5) × 10^{-3} s^{-1}. (B) Reconstitution of α_4-PDC·Mg^{2+} apoenzyme (0.05 mg mL^{-1}, 0.21 μM) with 10 mM TDP in the presence (●) or absence (○) of 100 mM pyruvamide in 100 mM sodium MES (pH 6.00) buffer containing 10 mM $MgSO_4$ at 30 °C. The line is drawn for reaction of 64 ± 10% of the α_4-PDC with k^2_{obsd} = (9.8 ± 4.4) × 10^{-2} s^{-1} and 36 ± 11% of the α_4-PDC with k^1_{obsd} = (8.7 ± 4.3) × 10^{-3} s^{-1}. The insets show replots of the data obtained in the fast (■) and slow phase (△) of the reconstitution reaction. The inset ordinates represent the logarithm of the activity of the more reactive PDC species corrected for the concentration of the slower reacting PDC species (Moore & Pearson, 1981). Note the difference in scale between panels A and B. The specific activities of 100%-reconstituted PDC were 60 U mg^{-1} (panel A) and 52 U mg^{-1} (panel B), respectively. The apoenzyme was preincubated with $MgSO_4$, and the reconstitution reaction was initiated by the addition of TDP (see text).

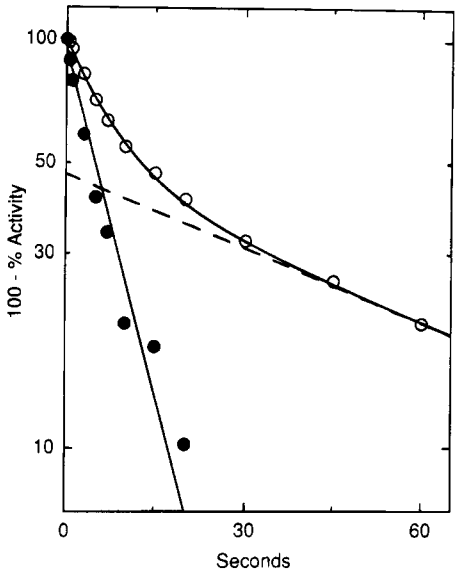


FIGURE 3: Reconstitution of mix-PDC·Mg^{2+} apoenzyme (9.0 mg mL^{-1}, 37.5 μM) with 10 mM TDP in the presence (●) or absence (○) of 150 mM pyruvamide in 150 mM sodium MES (pH 6.00) buffer containing 15 mM $MgSO_4$ at 30 °C. The apoenzyme was preincubated with $MgSO_4$, and the reconstitution reaction was initiated by the addition of TDP (see text). The line is drawn for a first-order rate constant of k_{obsd} = (1.5 ± 0.1) × 10^{-1} s^{-1} for reconstitution in the presence of pyruvamide (●). The other line is drawn for reaction of 53 ± 13% of the PDC with k^2_{obsd} = (1.4 ± 0.2) × 10^{-1} s^{-1} and 47 ± 13% of the PDC with k^1_{obsd} = (1.3 ± 0.3) × 10^{-2} s^{-1} for reconstitution in the absence of pyruvamide (○). The specific activity of 100%-reconstituted PDC was 60 U mg^{-1}. Note the difference in scale between Figures 2 and 3.

Figure 2 also shows that values of k_{obsd} are independent of TDP concentration in the range 10 mM (panel B)−100 mM (panel A) (corresponding to values of $[TDP]_{total}/[Mg^{2+}]_{total}$ in the range 1−13), which provides evidence that complex formation involving free TDP and Mg^{2+} does not significantly affect the values of k_{obsd} for reconstitution of apo-PDC·Mg^{2+} with TDP or apo-PDC·TDP with Mg^{2+} (see below). For example, under the reaction conditions of $[TDP]_{total}/[Mg^{2+}]_{total}$ = 0.005−200 used in these experiments (see below), estimated values of $[Mg^{2+}]_{free}/[TDP{\cdot}Mg^{2+}]$ in the range 0.01−200 were calculated from the specified total ionic concentrations of Na^+, Mg^{2+}, MES, and TDP in aqueous solution (Fabiato, 1988) with $K_{TDP{\cdot}Mg^-} \approx 1.48 \times 10^3$ M^{-1} and $K_{MES{\cdot}Mg^+} \approx 3.2$ M^{-1} in 100 mM sodium MES (pH 6.00) buffer at 30 °C.[7]

Figure 3 shows that reconstitution of apo-PDC·Mg^{2+} with saturating TDP is pyruvamide dependent under the reaction condition of ≤300:1 TDP:apo-PDC·Mg^{2+}. Reconstitution of apo-PDC·Mg^{2+} at this relatively low ratio of TDP:apo-PDC·Mg^{2+} exhibits simple first-order kinetics in the presence of saturating pyruvamide with k^2_{obsd} = (1.5 ± 0.1) × 10^{-1} s^{-1}. Under these same reaction conditions, bisphasic reconstitution kinetics are observed in the absence of pyruvamide with a burst [k^2_{obsd} = (1.4 ± 0.2) × 10^{-1} s^{-1}], which represents 53 ± 13% of the total reconstituted PDC activity, and a slow phase with an observed rate constant of k^1_{obsd} = (1.3 ± 0.3) × 10^{-2} s^{-1} for reconstitution of the remaining 47 ± 13% of the PDC activity.

[7] The apparent stability constants for TDP·Mg^- and MES·Mg^+ at 30 °C and pH 6.00 were estimated (Fabiato, 1988) with pK_a = 6.15 for MES, $K_{MES{\cdot}Mg^+}$ = 6.3 M^{-1}, and $K_{MES{\cdot}Na}$ = 1.41 × 10^6 M^{-1} (Dawson et al., 1986). We assume $K_{TDP{\cdot}Mg^-} = K_{ADP{\cdot}Mg^-} \approx 1.48 \times 10^3$ M^{-1} and pK_a values of 0.9, 2.0, and 6.0 for the diphosphate moiety in TDP (Dawson et al., 1986).

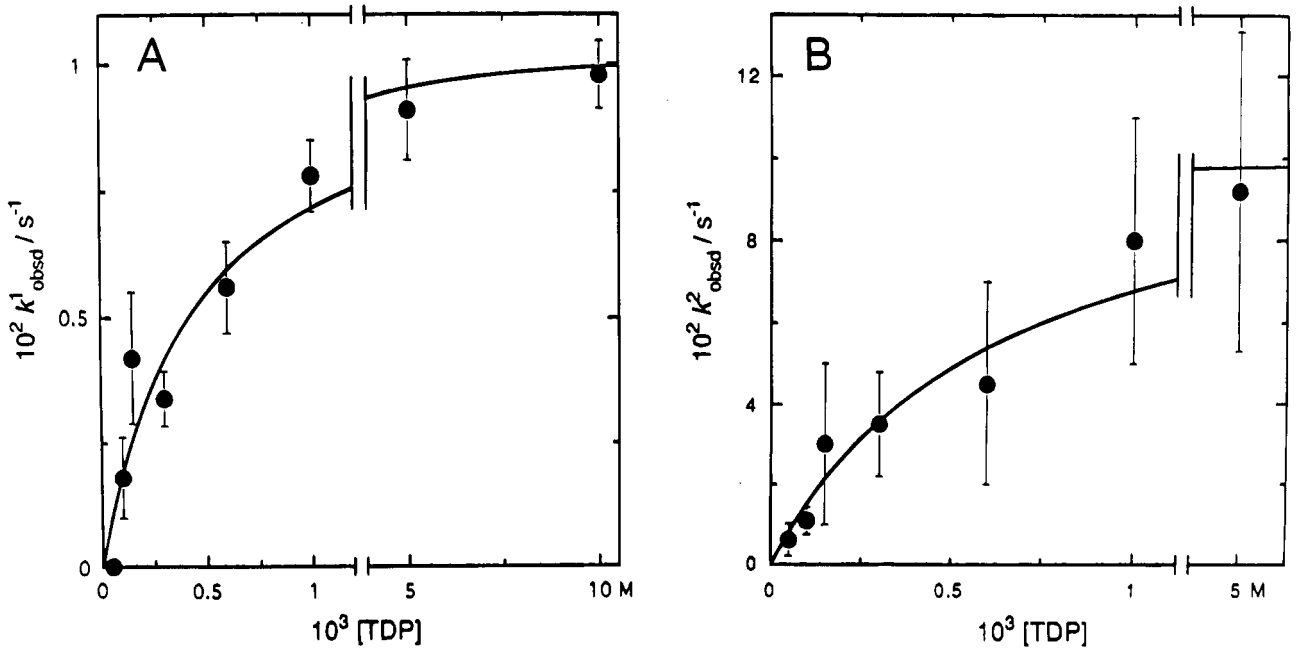


FIGURE 4: (A) Dependence of the observed rate constant k^1_{obsd} for reconstitution of mix-PDC·Mg^{2+} apoenzyme (0.06−0.12 mg mL^{-1}, 0.26−0.50 μM) on the total concentration of TDP in 100 mM sodium MES (pH 6.00) buffer containing 10 mM $MgSO_4$ at 30 °C. The line is drawn for an apparent equilibrium binding constant $K_{TDP} = 0.42 \pm 0.11$ mM and a first-order rate constant $k_1 = (1.0 \pm 0.3) \times 10^{-2}$ s^{-1} (see text). (B) Dependence of the observed rate constant k^2_{obsd} for reconstitution of mix-PDC·Mg^{2+} apoenzyme (0.06−0.12 mg mL^{-1}, 0.26−0.50 μM) on the total concentration of TDP in 100 mM sodium MES (pH 6.00) buffer containing 10 mM $MgSO_4$ at 30 °C. The line is drawn for an apparent equilibrium binding constant $K_{TDP} = 0.63 \pm 0.16$ mM and a first-order rate constant $k_2 = (1.1 \pm 0.6) \times 10^{-1}$ s^{-1} (see text). Note the difference in scale between panels A and B. The apoenzyme was preincubated with $MgSO_4$ in the absence of pyruvamide, and the reconstitution reaction was initiated by the addition of TDP (see text).

The observed first-order rate constants (k^1_{obsd} and k^2_{obsd}) for reconstitution of apo-PDC·Mg^{2+} with TDP (≥100:1 TDP:apo-PDC·Mg^{2+}) in the absence of pyruvamide increase over the range $k^1_{obsd} = 0.001-0.010$ s^{-1} and $k^2_{obsd} = 0.006-0.10$ s^{-1} with increasing total (= bound + free) TDP concentration in the range 0.05−1 mM and are independent of total TDP concentration in the range 1−10 mM with values of $k^1_{obsd} = (1.0 \pm 0.3) \times 10^{-2}$ s^{-1} and $k^2_{obsd} = (1.1 \pm 0.6) \times 10^{-1}$ s^{-1}, respectively (Figure 4). The decreasing concentration dependence of the values of k_{obsd} obtained at >1 mM TDP provides evidence for some type of complex formation between an apo-PDC·Mg^{2+} species and TDP at equilibrium ($K_{TDP} = 0.5 \pm 0.2$ mM) followed by rate-determining conversion to active holo-PDC with $k_1 = (1.0 \pm 0.3) \times 10^{-2}$ s^{-1} (Figure 4A) or $k_2 = (1.1 \pm 0.6) \times 10^{-1}$ s^{-1} (Figure 4B) [Jencks, 1987; see pp 19−36 of Johnson (1992)]. The apparent equilibrium binding constants K_{TDP} derived from steady-state kinetic measurements, 0.42 ± 0.11 and 0.63 ± 0.16 mM, are based on $[TDP]_{total}$ and are not significantly different from a previously reported value of 0.65 mM (Schellenberger, 1967; Schellenberger & Hübner, 1967). Experiments with ≥100:1 TDP:apo-PDC·Mg^{2+} (Figure 4) were carried out in the same manner as those shown for ≥1.6 × 10^4:1 TDP:apo-PDC·Mg^{2+} (Figure 2) with ≥8 time points. The relatively high degree of variation in k^2_{obsd} (Figure 4B) arises from difficulties in obtaining high precision for measurements of rapid reaction rates ($t_{1/2} \approx 7$ s) using the methods reported here.

Typical data for reconstitution of apo-PDC·TDP (apo-PDC preincubated with saturating TDP) with saturating Mg^{2+} in the absence of pyruvamide are shown in Figure 5. Under the reaction conditions of ≥1.2 × 10^4:1 Mg^{2+}:apo-PDC·TDP, the quantitative reconstitution of apo-PDC·TDP with Mg^{2+} to form holo-PDC exhibits first-order kinetics [$k^1_{obsd} = (1.3 \pm 0.4) \times 10^{-2}$ s^{-1}] in the absence or presence (data not shown) of saturating pyruvamide. Where multiple determinations of k^1_{obsd} were made, they agreed within ≤±16% of the average value.

The observed first-order rate constants (k^1_{obsd}) for reconstitution of apo-PDC·TDP with Mg^{2+} (≥20:1 Mg^{2+}:apo-PDC·TDP) in the absence of pyruvamide increase over the range $k^1_{obsd} = 0.001-0.016$ s^{-1} with increasing total (= bound + free) Mg^{2+} concentration in the range 0.001−1 mM and are independent of total Mg^{2+} concentration in the range 1−10 mM with a value of $k^1_{obsd} = (1.6 \pm 0.5) \times 10^{-2}$ s^{-1} (Figure 6). The decreasing concentration dependence of the values of k^1_{obsd} obtained at >1 mM Mg^{2+} provides evidence for some type of complex formation between apo-PDC·TDP and Mg^{2+} at equilibrium ($K_{Mg^{2+}} = 0.2 \pm 0.1$ mM) followed by rate-determining conversion to active holo-PDC with $k_1 = (1.6 \pm 0.5) \times 10^{-2}$ s^{-1} [Jencks, 1987; see pp 19−36 of Johnson (1992)]. The apparent equilibrium binding constant $K_{Mg^{2+}}$ derived from steady-state kinetic measurements, 0.21 ± 0.07 mM, is based on $[Mg^{2+}]_{total}$ and is similar to a previously reported value of $K_{Mg^{2+}} = 1.1$ mM (Schellenberger, 1967; Schellenberger & Hübner, 1967).

The observed first-order rate constant, k^2_{obsd}, for reconstitution of apo-PDC with TDP (≥30:1 TDP:apo-PDC) and Mg^{2+} (≥300:1 Mg^{2+}:apo-PDC) in the absence of pyruvamide increased with increasing [TDP] (0.01−5 mM) at fixed $[Mg^{2+}]_{total}$ (0.1, 0.5, or 10 mM) in the range $k^2_{obsd} = 3.0 \times 10^{-4}$ to 7.5×10^{-2} s^{-1} (Figure 7A). The dependence of k^2_{obsd} on [TDP] and $[Mg^{2+}]_{total}$ follows a rapid equilibrium ordered bireactant kinetic model involving binding of Mg^{2+} to apo-PDC followed by binding of TDP and formation of

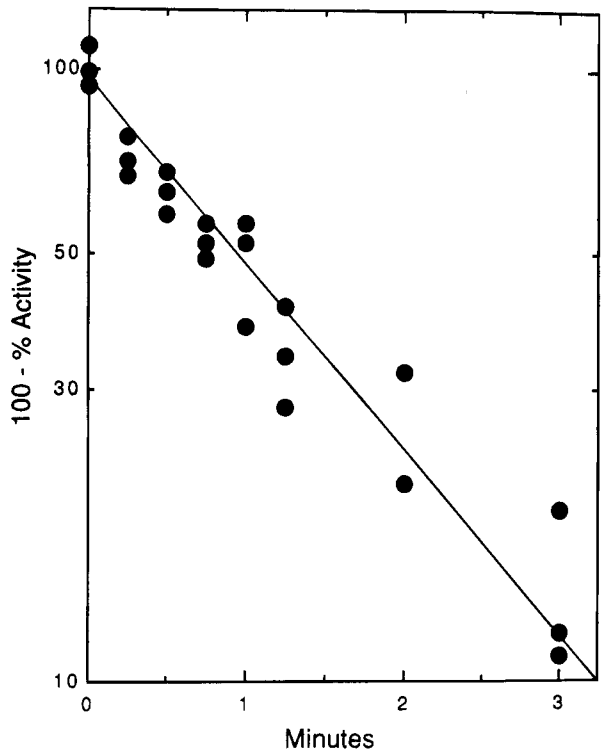


FIGURE 5: Reconstitution of mix-PDC·TDP apoenzyme (0.10 mg mL^{-1}, 0.43 μM) with 5 mM $MgSO_4$ in 100 mM sodium MES (pH 6.00) buffer containing 10 mM TDP at 30 °C. The apoenzyme was preincubated with TDP in the absence of pyruvamide, and the reconstitution reaction was initiated by the addition of $MgSO_4$. The line is drawn for a first-order rate constant of $k^1_{obsd} = (1.3 \pm 0.4) \times 10^{-2}$ s^{-1}. The specific activity of 100%-reconstituted PDC was 60 U mg^{-1}.

holo-PDC (Scheme 1) (Segal, 1975).

Scheme 1

$$^{a}E + \underset{+\ Mg^{2+}}{TDP} \underset{K_{Mg^{2+}}}{\rightleftharpoons} E_M \underset{K_{TDP}}{\rightleftharpoons} E^T_M \xrightarrow{k_2} {}^{h}E^T_M$$

$$\frac{1}{k^2_{obsd}} = \frac{A}{[TDP]} + \frac{1}{k_2} \quad (1)$$

$$A = \left(\frac{K_{TDP}K_{Mg^{2+}}}{k_2}\right)\frac{1}{[Mg^{2+}]} + \frac{K_{TDP}}{k_2} \quad (2)$$

Steady-state kinetic parameters were calculated from eq 1, which is the steady-state solution to Scheme 1; the term A (M s) represents the collection of rate and binding constants given in eq 2. The lines in Figure 7A are based on Scheme 1 using the rate law given in eq 1 with the following steady-state kinetic parameters: $A = (1.8 \pm 0.3) \times 10^{-1}$ M s (□), $A = (2.3 \pm 1.1) \times 10^{-2}$ M s (△), $A = (5.3 \pm 1.0) \times 10^{-3}$ M s (○), and $k_2 = (1.1 \pm 0.6) \times 10^{-1}$ s^{-1}. Figure 7B shows the dependence of the values of A on $[Mg^{2+}]_{total}$, and the line is based on Scheme 1 using eq 2 with $K_{TDP} = 0.5 \pm 0.2$ mM, $K_{Mg^{2+}} = 3 \pm 2$ mM, and $k_2 = (1.1 \pm 0.6) \times 10^{-1}$ s^{-1}. Values of k^1_{obsd} ($\leq 1 \times 10^{-3}$ s^{-1}) were too small to obtain reliable kinetic data under these reaction conditions.

DISCUSSION

Steady-State Kinetic Mechanism. Two different mechanisms have been proposed for the reconstitution of *S. carlsbergensis* apo-PDC with TDP and Mg^{2+}. Schellenberger and co-workers proposed random binding of TDP and Mg^{2+} to form a ternary complex with mix apo-PDC (^{a}E) at equilibrium followed by rate-determining "cyclization", k_c, to form active mix holo-PDC (^{h}E) (Scheme 2). The mechanism outlined in Scheme 2 was based on steady-state kinetic experiments and the observation of zero-order kinetics in [apo-PDC] under the reaction conditions of $\geq 1 \times 10^4$:1 TDP:apo-PDC·Mg^{2+} (Schellenberger, 1967; Schellenberger & Hübner, 1967; Nafe et al., 1972; Golbik et al., 1991). Estimated values of k_c have increased from 7.3×10^{-4} s^{-1} (Schellenberger, 1967; Schellenberger & Hübner, 1967) and 7.5×10^{-3} s^{-1} (Nafe et al., 1972) to ≥ 0.14 s^{-1} (Golbik et al., 1991). Single-turnover experiments on holo-PDC that required rapid and efficient reconstitution of apo-PDC with TDP and Mg^{2+} confirmed the observation that reconstitution was complete in ≤ 19 s ($k_c \geq 0.2$ s^{-1}) (Crane et al., 1993). An obligatory ordered reconstitution mechanism involving binding of free divalent metal followed by association of TDP was proposed on the basis of the absence of binding of a bidentate TDP·Rh^{3+} complex to mix apo-PDC from *S. carlsbergensis* (Lautens & Kluger, 1992). The results reported here (1) support a preferred-order mechanism involving two pathways for reconstitution of *S. carlsber-*

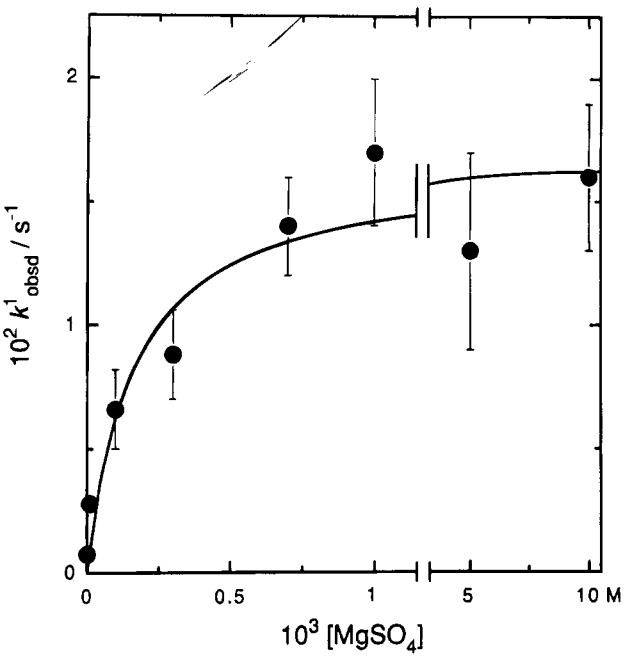
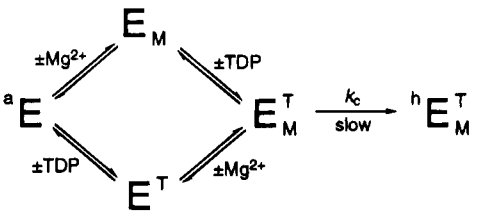


FIGURE 6: Dependence of the observed rate constant k^1_{obsd} for reconstitution of mix-PDC·TDP apoenzyme (0.07–0.10 mg mL^{-1}, 0.29–0.43 μM) on the total concentration of Mg^{2+} in 100 mM sodium MES (pH 6.00) buffer containing 10 mM TDP and 0–10 mM $MgSO_4$ at 30 °C. The apoenzyme was preincubated with TDP in the absence of pyruvamide, and the reconstitution reaction was initiated by the addition of $MgSO_4$. The line is drawn for an apparent equilibrium binding constant $K_{Mg^{2+}} = 0.21 \pm 0.07$ mM and a first-order rate constant $k_1 = (1.6 \pm 0.5) \times 10^{-2}$ s^{-1} (see text).

Scheme 2



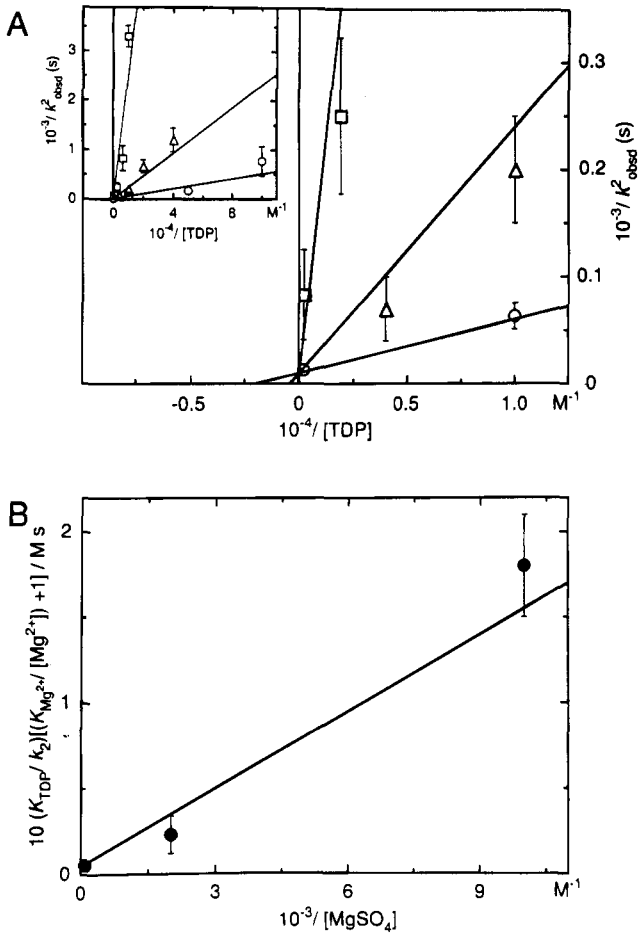
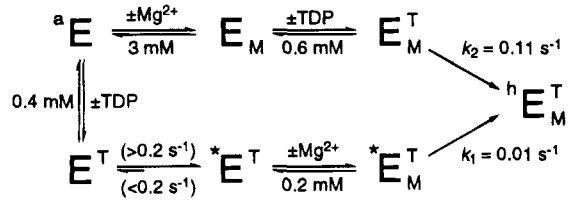


FIGURE 7: (A) Double-reciprocal plot showing the dependence of the observed rate constant k^2_{obsd} for reconstitution of mix-PDC apoenzyme (0.04−0.08 mg mL^{-1}, 0.17−0.33 μM) in the absence of pyruvamide on the concentration of TDP (0.01−5 mM) in 100 mM sodium MES (pH 6.00) buffer at 30 °C containing 0.1 mM (□), 0.5 mM (△), or 10 mM (○) $MgSO_4$ (see text). The inset shows the abscissa and ordinate intercepts at high TDP concentration. The slope of the plot against 1/[TDP] gives $A = (K_{TDP}/k_2)[(K_{Mg^{2+}}/[Mg^{2+}]) + 1]$ (see text). (B) Replot showing the dependence of A on $1/[Mg^{2+}]_{total}$. The line is based on Scheme 1, using the rate law given in eqs 1−2 and with $K_{TDP} = 0.5 \pm 0.2$ mM, $K_{Mg^{2+}} = 3 \pm 2$ mM, and $k_2 = (1.1 \pm 0.6) \times 10^{-1}$ s^{-1} (see text).

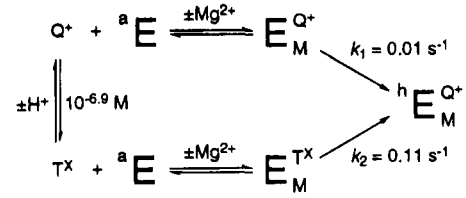
Scheme 3



gensis apo-PDC and (2) confirm and extend the conclusion that reconstitution of *S. carlsbergensis* apo-PDC is rapid and complete under certain reaction conditions.

Scheme 3 shows a steady-state mechanism for apo-PDC reconstitution with TDP and Mg^{2+} that is consistent with the results of steady-state kinetic experiments. The curves in Figures 2−7 agree with curves simulated by numerical integration with rate and equilibrium constants summarized in Scheme 3 using the program KINSIM (Barshop et al., 1983). Scheme 3 is a minimal mechanism for holo-PDC formation, and a preferred-order [rather than a completely random mechanism (Scheme 2)] is implied; the proposed

Scheme 4



mechanism confirms and extends components of previous mechanisms. The mechanistic details in Scheme 3 are based on the following results.

(1) The observation of biphasic kinetics requires two pathways for reconstitution of apo-PDC·Mg^{2+} with TDP with each pathway defined by a single rate process. The deviation from a simple first-order plot for the reconstitution of apo-PDC (Figure 2) provides evidence for two parallel first-order reactions producing a common product (holo-PDC) (Moore & Pearson, 1981). Such reactions, of course, take place independently as far as the reactants (apo-PDC species) are concerned. Kinetic modeling shows that simple first-order kinetics is expected for mechanisms involving a single pathway for reconstitution of apo-PDC, including an obligatory ordered reaction mechanism (see above) or Scheme 2. We conclude that the observation of biphasic kinetics excludes these and other mechanisms involving a single pathway for reconstitution of apo-PDC.

The "burst" shown in Figure 2 most likely represents the presence of different apo-PDC species because the burst is independent of (1) the identity or presence of PDC isozymes (Figure 2) and (2) trace divalent metal or TDP remaining in the apo-PDC preparations. The reconstitution of apo-PDC involves the reaction of stable tetrameric apo-PDC to form tetrameric holo-PDC (Gounaris et al., 1975; Hübner et al., 1990). Monomeric or dimeric apo-PDC is not observed under the reaction conditions of pH ≤6.2 (König et al., 1992), which provides evidence against oligomerization of apo-PDC or reconstitution involving apo-PDC dimers. Kinetic modeling requires that the less reactive component represents 50% and the more reactive component 50% of the original mixture, which suggests approximately 50:50 partitioning of apo-PDC species between the two pathways (Figure 2). These results show that PDC isozymes do not differ significantly with respect to the mechanism of reconstitution with TDP and Mg^{2+}. This is important because the major isozymes (α_4 and $\alpha'_2\beta_2$) are kinetically and mechanistically different on the basis of catalysis of acetoin formation from acetaldehyde (Stivers & Washabaugh, 1993). The [PDC]-independent burst (Figures 2 and 3) provides evidence that a rate-limiting conformational step is being observed.

The presence of two apo-PDC species and biphasic reconstitution kinetics cannot be attributed to the binding of alternate forms of TDP (T^X) present at equilibrium in aqueous solution (Scheme 4). In aqueous solution, TDP can exist as a thiazolium ion (Q^+), a neutral pseudobase derived from nucleophilic attack at C(2) of the thiazolium ring by hydroxide ion or water (T^o), a neutral tricyclic species formed by intramolecular nucleophilic addition of the exocyclic 4′-amino group to the C(2) position of the thiazolium ring (T^N), and enethiolate species derived from T^o and T^N by reversible ring opening of the thiazolium ring. Q^+ and T^N have been proposed to bind to the soluble receptor component of *Escherichia coli* periplasmic thiamin permease (Hollenbach,

1995). Q^+, T^o, and T^N interconvert in rapid pH dependent equilibria in aqueous solution with an apparent pK_a value of 6.9 ± 0.1 (Washabaugh et al., 1993). N(1′)-protonated Q^+ ($pK_a^{N(1')} = 5.1$) (10%), free Q^+ (80%), T^o (≤1%), and T^N (10%) are present in aqueous solution at pH 6.0 (Hollenbach, 1995). Identical biphasic kinetics is observed for reconstitution of apo-PDC·Mg^{2+} with TDP at pH 6.4 (data not shown) where N(1′)-protonated Q^+ (5%), free Q^+ (70%), T^o (≤1%), and T^N (25%) are present in aqueous solution at pH 6.4. We conclude that Q^+ species are the major forms of TDP present under the reaction conditions (T^o and T^N are minor forms and designated T^X in Scheme 4) and that the relative distribution of the two reactive apo-PDC species (50:50 as determined by kinetic modeling; see Figures 2 and 3) is independent of the relative distribution of Q^+ species. This excludes a single apo-PDC species binding multiple forms of TDP at equilibrium (Scheme 4). The presence of two apo-PDC species also cannot be attributed to a pyruvate (substrate)- or pyruvamide (allosteric effector)-dependent pathway (Schellenberger, 1967; Schellenberger & Hübner, 1967) because the biphasic kinetics was independent of the presence or absence of the substrate or allosteric effector (Figure 2) (see below).

(2) The presence of two pathways is further supported by a change to a single pathway—a change from biphasic to simple first-order kinetics—with a change in the order of cofactor addition (Figure 5). The observation of simple first-order kinetics for reconstitution of apo-PDC·TDP with Mg^{2+} also provides evidence that existence of the two apo-PDC species depends on the order of cofactor addition before reconstitution to form a single holo-PDC species.

The fact that only the slower rate constant is observed for reconstitution of apo-PDC·TDP with Mg^{2+} suggests that the less reactive apo-PDC species is apo-PDC·TDP and that biphasic kinetics is observed because of a first-order process that mediates a change in the state of apo-PDC·TDP (E·TDP → *E·TDP; see Scheme 3). We assume that the k_2 pathway is much less favorable than the k_1 pathway under these reaction conditions because of a shift in the equilibrium concentration of the two apo-PDC species toward the less reactive component ($k^2_{obsd} \neq k^1_{obsd}$). We also assume that k_2 is independent of the order of cofactor addition ($k_2 \neq k_1$).

(3) The decreasing dependence of the values of k^1_{obsd} and k^2_{obsd} on [TDP] (Figure 4) and k^1_{obsd} on $[Mg^{2+}]_{total}$ (Figure 6) provides evidence for formation of an intermediate(s) at equilibrium followed by rate-determining first-order formation of holo-PDC. More specifically, these results provide evidence for reaction (at equilibrium) of apo-PDC·Mg^{2+} with TDP to form a ternary apo-PDC(Mg^{2+})(TDP) complex (Figure 4B), TDP binding to free apo-PDC (Figure 4A), and Mg^{2+} binding to a different apo-PDC·TDP species (*E·TDP) to form a different ternary apo-PDC(Mg^{2+})(TDP) complex (Figure 6). We conclude that partitioning of the two apo-PDC species between the reconstitution pathways reflects the ratio of K_{TDP} and $K_{Mg^{2+}}$.

(4) Standard methods for determination of the order of addition of the cofactors, such as slope intercept analysis using double-reciprocal plots (Segal, 1975) (Figure 7), provide evidence that cofactor addition occurs by a rapid equilibrium ordered mechanism (Mg^{2+} binding followed by TDP) in the k_2 pathway for this bireactant system (see Scheme 1).

(5) Values of k^1_{obsd} and k^2_{obsd} are independent of $[Mg^{2+}]_{free}$/[TDP·Mg^{2+}], which provides evidence that either free TDP, free Mg^{2+}, or TDP·Mg^{2+} can bind to apo-PDC. Previous studies involving a stable TDP·Rh^{3+} bidentate complex suggested that a preformed complex would not be able to enter the TDP-binding site in apo-PDC if the site remains unchanged (Lautens & Kluger, 1992); however, interpretation of this experiment is complicated by residual Mg^{2+} in the apo-PDC. We conclude that complex formation between TDP and Mg^{2+} in solution does not significantly affect reconstitution of apo-PDC with these cofactors, and, consequently, free species are shown in Scheme 3. This conclusion implies that (1) labile TDP·Mg^{2+} changes to a structure similar to TDP·Rh^{3+} after binding to apo-PDC or (2) stable TDP·Rh^{3+} cannot be used as a model for binding of relatively labile TDP·divalent metal complexes to apo-PDC. Although the TDP·Rh^{3+} complex and TDP·Mg^{2+} complex determined by X-ray crystallographic analysis of the holoenzyme form of α_4-PDC are bidentate (Dyda et al., 1993), other structural similarities have not been strictly defined.

The proposed mechanism (Scheme 3) for reconstitution of *S. carlsbergensis* apo-PDC is similar to the random mechanism for reconstitution of *Zymomonas mobilis* apo-PDC involving two equivalent pathways for reconstitution [(1) TDP followed by Mg^{2+} or (2) Mg^{2+} followed by TDP] with partially rate-determining conformational changes upon cofactor binding to apo-PDC (Diefenbach & Duggleby, 1991).

Effects of Substrate and Allosteric Effector. The activation of PDC by its substrate (pyruvate) occurs at an allosteric site, and pyruvamide, which is not a substrate or a competitive inhibitor, activates the enzyme completely without a detectable lag time on the pre-steady state (millisecond) time scale (Hübner et al., 1970, 1978; Crane et al., 1993). The allosteric effector (pyruvate or pyruvamide) adds rapidly to a cysteine sulfhydryl group in the enzyme regulatory site, followed by a slow, rate-determining isomerization step of the substrate−PDC adduct [see footnotes 7 and 18−20 in Alvarez et al. (1991); Baburina et al., 1994]. Allosteric activation occurs, in part, by the increasing of the rate constant for acetaldehyde release from PDC (Stivers & Washabaugh, 1993). It was important to know what effect, if any, pyruvamide has on the rate of reconstitution of holoenzyme since strong inhibition of reconstitution would make some single-turnover experiments with pyruvamide-activated PDC impossible [see, for example, Crane et al. (1993)].

Both pyruvate and pyruvamide have significant effects on apo-PDC reconstitution with TDP and Mg^{2+}. The rate constants for apo-PDC reconstitution are independent of [pyruvamide] in the range 0−150 mM at [apo-PDC] ≤ 37.5 μM (Figures 2 and 3). At [apo-PDC] > 37.5 μM, the presence of saturating (150 mM) pyruvamide strongly favors the k_2 (Mg^{2+} followed by TDP) pathway (Scheme 3)—the k_1 (TDP followed by Mg^{2+}) pathway is not observed in the presence of saturating pyruvamide—with no effect on the value of k_2; as a consequence, reconstitution is complete in ≤19 s. We do not have a satisfactory explanation why the effect of pyruvamide occurs only at relatively high [PDC]. A change in the rate-determining step for acetaldehyde formation from pyruvate or some type of complex formation

involving reactants at relatively high [PDC] (Crane et al., 1993) provides precedence that may be relevant.

Pyruvate decreases the value of k_2 ≤3-fold and has no effect on the value of k_1. We conclude that the presence of pyruvate strongly favors the k_2 (Mg^{2+} binding followed by TDP) pathway (Scheme 3). The k_1 (TDP followed by Mg^{2+}) pathway is unaffected by the presence of saturating (≥30 mM) pyruvate. We suggest that pyruvate interferes with a conformational change(s) required for holo-PDC formation via the k_2 pathway. Binding of pyruvate (Nafe et al., 1972) or pyruvamide (Hübner et al., 1990) induces a conformational change(s) in holo-PDC and provides precedence that may be relevant. A 10-fold rate-enhancing effect of substrate (pyruvate) was reported under significantly different reconstitution reaction conditions (Schellenberger, 1967; Schellenberger & Hübner, 1967); however, interpretation of these reconstitution experiments is complicated by allosteric activation of holo-PDC (Hübner et al., 1970; Golbik et al., 1991; Baburina et al., 1994).

Conclusions. Reconstitution of the major apo-PDC isozymes from *S. carlsbergensis* with TDP and Mg^{2+} exhibits biphasic kinetics, which provides evidence for two pathways, TDP binding followed by Mg^{2+} (k_1) or Mg^{2+} binding followed by TDP (k_2), in a rapid equilibrium preferred-order ($k_2 > k_1$) mechanism. This conclusion is supported by a change in reconstitution pathway with the order of cofactor addition, which is inconsistent with a single pathway involving ordered binding of the metal ion followed by TDP. The presence of pyruvamide has no significant effect on the rate constants for apo-PDC reconstitution and favors the k_2 pathway; pyruvate decreases the value of k_2 ≤3-fold and has little or no effect on the value of k_1. We suggest that substrate interferes with a conformational change(s) required for holo-PDC formation via the k_2 pathway. These results are summarized in a model for apo-PDC reconstitution.

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