Direct Observation of the Kinetic Fate of a Thiamin Diphosphate Bound Enamine Intermediate on Brewers' Yeast Pyruvate Decarboxylase. Kinetic and Regiospecific Consequences of Allosteric Activation

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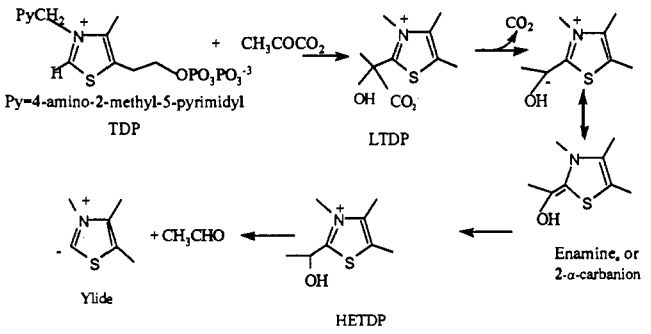
Abstract: The interaction of (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid with brewer's yeast pyruvate decarboxylase was examined by kinetic and absorption spectroscopic means. The compound undergoes catalytic turnover, leading to a thiamin diphosphate bound enamine intermediate that can be protonated at both allylic positions leading to *p*-methylcinnamaldehyde and *p*-methyldihydrocinnamic acid in a ratio of 1:3 in the absence of and 3:2 in the presence of the allosteric activator pyruvamide. The compound is also a weak but irreversible inactivator of the enzyme, and according to colorimetric titration of the enzyme prior to and subsequent to inactivation, it reacts with a Cys side chain with a 1:1 stoichiometry; i.e., one Cys/subunit is being modified. The data are consistent with partitioning of the 2-(*p*-methyldihydrocinnamoyl)thiamin diphosphate between nonenzymic hydrolysis to the free *p*-methyldihydrocinnamic acid and transfer to a Cys on the enzyme forming a *p*-methyldihydrocinnamoyl thiol ester. The latter at the pH used is quite stable, and hence inactivates the enzyme. An enzyme-bound enamine intermediate can be detected at 440 nm, and its rates of formation and disappearance can be conveniently monitored. A thiazolium model for a precursor to such an enamine, 2-[γ-[tetrahydro-2*H*-pyran-2-yl)oxy]-(*E*)-cinnamyl]-3,4,5-trimethylthiazolium ion, when treated with $(TMS)_2NNa$ in Me_2SO gave an absorbance with identical λ_{max}, confirming the assignment of the absorbance observed from such conjugated 2-keto acids to the thiamin-bound enamine structure. Finally, the allosteric activator pyruvamide was found to enhance the rate of enamine formation by as much as 50-fold, both the rates of formation and conversion to product of the enamine being affected by the allosteric regulation. On the basis of data provided, (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid is proposed as a convenient active-site titrant for pyruvate decarboxylase.

Introduction

Thiamin diphosphate (TDP) is a coenzyme of very great importance in mammalian carbohydrate metabolism.[1] While its functions include both nonoxidative and oxidative chemistry, the simplest reaction catalyzed by TDP is pyruvate decarboxylation in yeast (Scheme I).[2]

According to this pathway, there are three covalent intermediates between the coenzyme and the substrate/product. The first (lactyl-TDP, LTDP)[3] and third (hydroxyethyl-TDP, HETDP)[4] of these have been synthesized chemically. This laboratory has for several years been engaged in research leading to an elucidation of the properties of the second of these intermediates, the enamine or 2α-carbanion both in models[5] and on brewer's yeast pyruvate decarboxylase.[6] The enamine intermediate (at its C2α atom) is probably the most versatile one of the three covalent intermediates: It is capable of being protonated to yield 2-(1-hydroxyethyl)-TDP (HEDTP) that is decomposed to acetaldehyde. It can be oxidized by lipoamide (as in all α-keto acid dehydrogenases), by flavin (as in pyruvate oxidase), or by an Fe_4S_4 cluster (as in pyruvate-ferredoxin oxidoreductase). Finally, it can react as a nucleophile as in the transketolases and in the acetohydroxyacidsynthetases (important in branched-chain amino acid metabolism).

Scheme I. Mechanism of PDC



Some time ago we reported that brewer's yeast pyruvate decarboxylase (PDC) can process (*E*)-2-oxo-4-phenyl-3-butenoic acids that have a variety of substituents on their phenyl rings.[6a-d,h] Several among these (those that carry a strong electron-withdrawing substituent) turned out to be mechanism-based inactivators of the enzyme, while others are simply poor substrates. Those that turned out to be inhibitory were also shown to give rise to a new absorbance centered at 430–440 nm, a λ_{max} that we had attributed to the enamine intermediate present both on the inhibitory and on the substratelike turnover pathway.[6b,c] More recently, Annan et al. showed that the *p*-bromomethyl analogue is a reversible inhibitor (the inhibition does not persist), whereas the *p*-chloromethyl analogue is an irreversible inactivator of PDC.[6g] Both analogues were found to also release halide ion concomitant with the inhibition, implying the intermediacy of the enamine that lost halide to give a quinone methide (Scheme II). The latter would rearrange to 2-acyl-TDP, which would subsequently be hydrolyzed to free enzyme. As a control, the *p*-methyl analogue was also studied and was found to be a slow substrate with weak, if any, inhibitory properties under the conditions employed.

In the meantime, our efforts to generate structures analogous to the enamine in a quantitative fashion bore fruit. Based on a

(1) For leading references see: (a) Krampitz, L. O. *Annu. Rev. Biochem.* **1969**, *38*, 213-240. (b) Krampitz, L. O. *Thiamin Diphosphate and Its Catalytic Functions*; Marcel Dekker: New York, 1970. (c) Sable, H. Z., Gubler, C. J., Eds. *Ann. N.Y. Acad. Sci.* **1982**, *378*, 7-122. (d) Kluger, R. *Chem. Rev.* **1987**, *87*, 863-876. (e) Schellenberger, A., Schowen, R. L., Eds. *Thiamin Pyrophosphate Biochemistry*; CRC Press: Boca Raton, FL, 1988; Vol. 1.

(2) Breslow, R. *J. Am. Chem. Soc.* **1958**, *80*, 3719-3726.

(3) Kluger, R.; Smyth, T. *J. Am. Chem. Soc.* **1981**, *103*, 1214-1216.

(4) Krampitz, L. O.; Votaw, R. *Methods Enzymol.* **1966**, *9*, 65-60.

(5) (a) Jordan, F.; Kudzin, Z. H.; Rios, C. B. *Stud. Org. Chem.* **1987**, *31*, 461-468. (b) Jordan, F.; Kudzin, Z. H.; Rios, C. B. *J. Am. Chem. Soc.* **1987**, *109*, 4415-4416. (c) Bordwell, F. G.; Satish, A. V.; Rios, C. B.; Chung, A. C.; Jordan, F. *J. Am. Chem. Soc.* **1990**, *112*, 792-797. (d) Barletta, G.; Chung, A. C.; Rios, C. B.; Jordan, F.; Schlegel, J. M. *J. Am. Chem. Soc.* **1990**, *112*, 8144-8149.

(6) (a) Kuo, D. J.; Jordan, F. *Biochemistry* **1983**, *22*, 3735-3740. (b) Kuo, D. J.; Jordan, F.; *J. Biol. Chem.* **1983**, *258*, 13415-13417. (c) Jordan, F.; Adams, J.; Farzami, F.; Budzin, Z. H. *J. Enzyme Inhib.* **1986**, *1*, 139-149. (d) Kuo, D. J.; Dikdan, G.; Jordan, F. *J. Biol. Chem.* **1986**, *261*, 3316-3319. (e) Jordan, F.; Kudzin, Z. H.; Kuo, D. J. *Ann. N. Y. Acad. Sci.* **1986**, *471*, 308-309. (f) Jordan, F.; Akinoyosoye, O.; Dikdan, G.; Kudzin, Z. H.; Kuo, D. J. In *Thiamine Pyrophosphate Biochemistry*; Schellenberger, A., Schowen, R. L., Eds.: CRC Press: Boca Raton, FL, 1988; Vol. 1, pp 79-92. (g) Annan, N.; Paris, R.; Jordan, F. *J. Am. Chem. Soc.* **1989**, *111*, 8895-8901. (h) Annan, N.; Jordan, F. *J. Am. Chem. Soc.* **1990**, *112*, 3222-3223.

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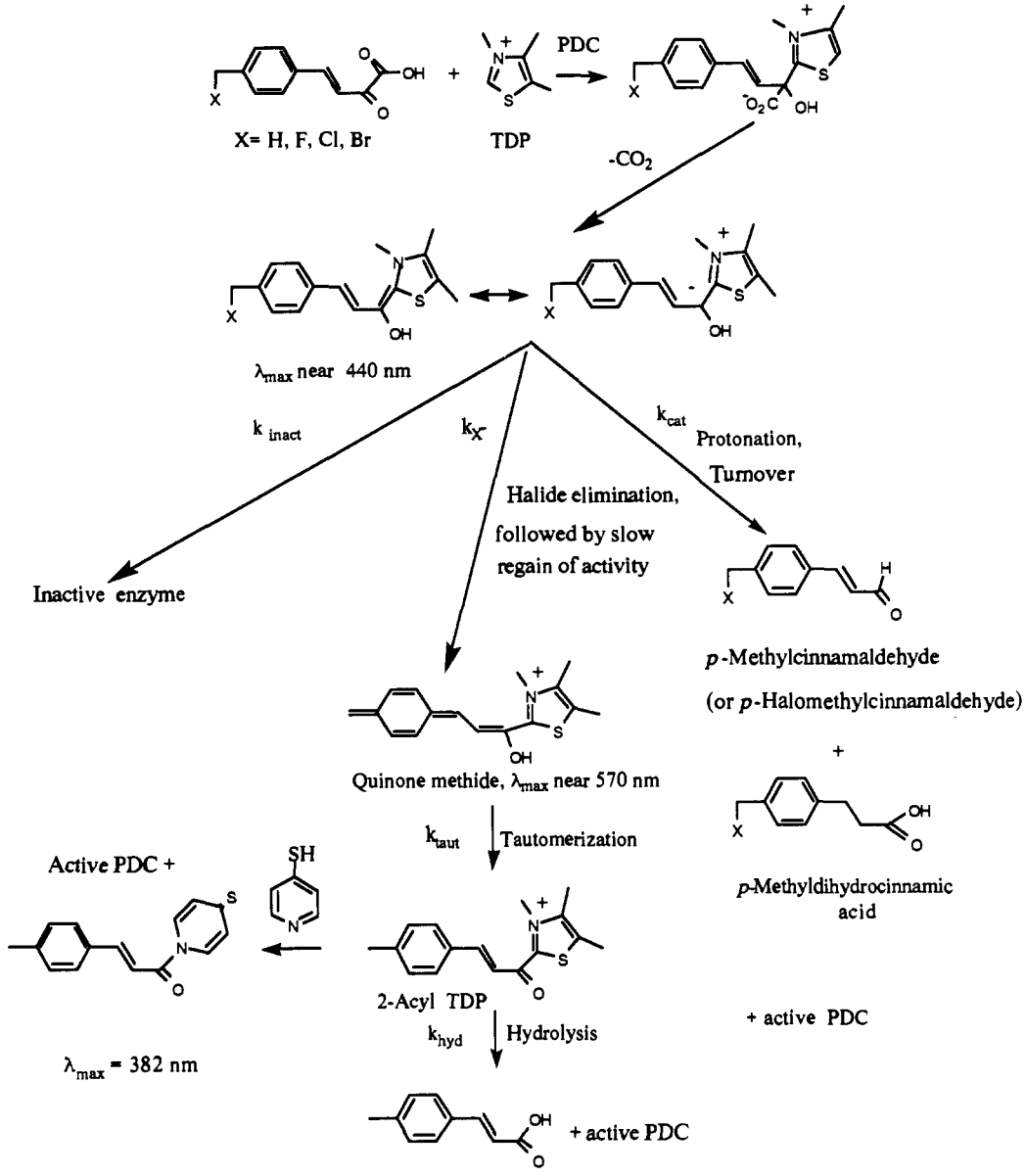
Scheme II. Reactions of (*E*)-2-Oxo-4-phenyl-3-butenoic Acids with Pyruvate Decarboxylase



report by Rastetter and co-workers,[7] 2-(1-hydroxyalkyl)thiazole was synthesized and then alkylated first at oxygen and then at nitrogen. The addition of a strong base such as potassium *tert*-butoxide or $(TMS)_2NNa$ in an aprotic solvent enabled us to generate the C2α-ionized conjugate base and to determine its structure,[5a,b] conjugate acidity,[5c] and redox properties.[5d]

In order to gain further insight to the conjugation between the thiazolium ring and the sp^2-hydridized C2α atom, 2-[γ(γ-hydroxy)cinnamyl]thiazole was synthesized and converted to the O-protected *N*-methylthiazolium salt **4** (2-[γ-[(tetrahydro-2*H*-pyran-2-yl)oxy]-(*E*)-cinnamyl]-3,4,5-trimethylthiazolium ion). The synthesis and UV–vis spectrum of the enamine generated from the latter are reported here, the visible spectrum supports the assignment of the absorbance on PDC to the enamine.[6b,c]

$$p\text{-}CH_3C_6H_4CH{=}CHCOCO_2H \quad \mathbf{1}$$

$$p\text{-}CH_3C_6H_4CH_2CH_2CO_2H \quad \mathbf{2} \qquad p\text{-}CH_3C_6H_4CH{=}CHCHO \quad \mathbf{3}$$

Most importantly, we also report here that the decarboxylation of (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid (**1**) is accompanied by the appearance of a new absorption centered at 440 nm as well. The time course of the formation and disappearance of this enzyme-bound intermediate can now be studied directly. The time course of the behavior of this intermediate and of substrate consumption and product appearance demonstrates that the intermediate is catalytically competent with the overall reaction and lead us to conclude with confidence for the first time that the enamine is indeed on the reaction pathway of PDC for this slow substrate/inhibitor.

Experimental Section

General Procedures. NMR spectra were recorded on an IBM WP 200 SY or Varian VXR 400 spectrometer. Chemical shifts are reported in δ values (from tetramethylsilane). All chemicals were of the highest purity commercially available.

Synthesis. 2-(γ-Hydroxy-(*E*)-cinnamyl)-4,5-dimethylthiazole. *n*-BuLi (8.0 mL, 10 M, 80 mmol) was suspended in anhydrous THF (20 mL) under Ar and was cooled to −77 °C. 4,5-Dimethylthiazole (Pyrazine Specialties; 9.0 g, 80 mmol), which was dissolved in THF (20 mL), was introduced in portions via a needle/septum device under Ar over a period of 15–20 min. The reaction mixture was stirred for approximately 40–45

(7) Rastetter, W. H.; Adams, J. W.; Nummey, L. J.; Frommer, J. E.; Roberts, K. B. *J. Am. Chem. Soc.* **1979**, *101*, 2752–2753.

min after which *trans*-cinnamaldehyde (10.6 g, 80 mmol) dissolved in THF (20 mL) was added in portions under the same conditions. After the mixture was stirred for 1 h, EtOH/H_2O was added while the temperature was maintained between −4 and −15 °C. The solvent was then removed at a rotary evaporator, and the dried residue was extracted with ether. The ether was then evaporated, and the crude product was chromatographed on a silica gel column with a mixture of hexane/ether as eluent. Recrystallization from hexane/ether yielded 8.8 g (36 mmol, 45%) of the desired product. ^{1}H NMR (400 MHz, $CDCl_3$/TMS): δ 7.48–7.12 (m, 5 H, C_6H_5), 6.7 (d, 1 H, J = 15.6 Hz), 6.4 (dd, 1 H, J = 7, 15.6 Hz), 5.5 (dd, 1 H, J = 5.5, 7 Hz; collapsed to d, J = 7 Hz when treated with a drop of D_2O), 3.15 (d, 1 H, J = 5.5 Hz; disappeared when treated with D_2O), 2.40 (s, 3 H), 2.25 (s, 3 H). Anal. Calcd for $C_{14}H_{15}NOS$: C, 68.54; H, 6.16; N, 5.71; S, 13.07. Found: C, 68.51; H, 6.16; N, 5.68; S, 13.10. MS (m/z, 70 eV): 245 (M^+, 36%).

2-[γ-[(Tetrahydro-2*H*-pyran-2-yl)oxy]-(*E*)-cinnamyl]-4,5-dimethylthiazole (*d,l* Mixtures of Two Diastereomers). 2-(γ-Hydroxy-(*E*)-cinnamyl)-4,5-dimethylthiazole (100 mg, 4.08 mmol), dihydropyran (686 mg, 8.16 mmol), and *p*-toluenesulfonic acid (39 mg, 0.20 mmol) were dissolved, and the mixture was heated at reflux in anhydrous THF (20 mL) under Ar for ca. 3.5–4 h until the reaction was completed. Next, the solvent was removed at a rotary evaporator, and the dried crude product was chromatographed on a silica gel column, eluting with hexane/ether as eluant, giving a purified racemic mixture of diastereomeric products in 67.6% yield (910 mg, 2.76 mmol). ^{1}H NMR (400 MHz, $CDCl_3$/TMS): δ 7.6–6.9 (m, 10 H), 6.75 (d, 2 H, J = 16 Hz), 6.48 (dd, 1 H, J = 7, 16 Hz), 6.15 (dd, 1 H, J = 7, 16 Hz), 5.72 (d, 1 H, J = 7 Hz), 5.42 (d, 1 H, J = 7 Hz), 4.90 (t, 1 H, J = 7.0 Hz), 4.78 (t, 1 H, J = 7.0 Hz), 3.9 (m, 2 H), 3.5 (m, 2 H), 2.5 (s, 6 H), 2.25 (s, 6 H), 2.0–1.3 (m, 12 H). Anal. Calcd for $C_{19}H_{23}NO_2S$: C, 69.27; H, 7.03; N, 4.25; S, 9.73. Found: C, 68.99; H, 7.06; N, 4.30; S, 9.82. (+) FAB: m/z 329.7 (M^+, 20%).

2-[γ-[(Tetrahydro-2*H*-pyran-2-yl)oxy]-(*E*)-cinnamyl]-3,4,5-trimethylthiazolium Tetrafluoroborate (4). 2-[γ-[(Tetrahydro-2*H*-pyran-2-yl)oxy]-(*E*)-cinnamyl]-4,5-dimethylthiazole (100 mg, 0.304 mmol, racemates of diasteromers from above) was dissolved in methylene chloride (10 mL), and the mixture was cooled to 0 °C. Trimethyloxonium tetrafluoroborate (45 mg, 0.30 mmol) was added, and the mixture was stirred under Ar for ca. 16–20 h. The solid product was collected by filtration, and the residue was washed with anhydrous ether and dried. Next, the product was recrystallized from MeOH/ether, yielding 100 mg of **4** (0.23 mmol, 78% yield of a mixture of two pairs of diastereomeric racemates). ^{1}H NMR (400 MHz, CD_3CN/TMS): δ 7.60–7.30 (m, 10 H), 7.00 (d, 1 H, J = 15.6 Hz), 6.90 (d, 1 H, J = 15.6 Hz), 6.40 (dd, 1 H, J = 7.0, 16.0 Hz), 6.20 (dd, 1 H, J = 7.0, 16.0 Hz), 5.90 (d, 1 H, J = 7.0 Hz), 5.80 (d, 1 H, J = 7.0 Hz), 4.90 (t, 1 H, J = 7.0 Hz), 4.80 (t, 1 H, J = 7.0 Hz), 4.00 (s, 3 H), 3.90 (s, 3 H), 3.80 (m, 2 H), 3.60 (m, 2 H), 2.45 (s, 3 H), 2.40 (two closely spaced singlets, 3 H each), 2.30 (s, 3 H), 1.90–1.65 (m, 6 H), 1.60–1.30 (m, 6 H). Anal. Calcd for $C_{20}H_{26}NO_2SBF_4 \cdot H_2O$: C, 53.46; H, 5.83; N, 3.12; S, 7.14. Found: C, 53.90; H, 5.85; N, 3.54; S, 7.20. (+) FAB m/z 343.7 (M^+, 78%).

Compound **1** was synthesized as reported earlier.[6g]

Enzyme Purification, Assay, and Inhibition. Initially, the enzyme was purified according to a procedure reported by Kuo et al.[6d] More recently, the enzyme purification protocol developed by Farrenkopf in this laboratory, capable of separating several pure homo- and heterotetrameric active fractions, was employed. Protein assay was performed with use of the Biorad kit,[8] while the enzyme activity was assayed by the pH-stat method.[9] One unit of activity is defined as the amount of PDC required to convert 1 μmol of pyruvate to acetaldehyde per minute at 30 °C.

Steady-State Kinetics. Michaelis–Menten plots were developed for the rate of disappearance of **1** at 20 °C. The typical reaction mixture contained variable amounts of **1**, 20 units of PDC, and 1 mM TDP and $MgCl_2$ in 0.1 M citrate buffer, pH 6.0, with 20% (v/v) ethylene glycol in 1-mL total volume. The disappearance of **1** was monitored at 380 nm (ϵ_{380} = 600 M^{-1} cm^{-1}) at 30-s intervals for 150 s. Absorbance vs time plots were linear.

Time-Dependent Inactivation Kinetics. In a typical experiment 35 units of holo-PDC was incubated with variable amounts of compound **1** and 1 mM TDP and $MgCl_2$ in 0.1 M citrate, pH 6.0, with 20% (v/v) ethylene glycol at 25 °C in the absence and presence of 80 mM pyruvamide. At the indicated times aliquots were removed for pH-stat assay.

pH Dependence of Inactivation of Holo-PDC with Compound 1. Forty units of holo-PDC was incubated with 5 mM **1** and 1 mM TDP and $MgCl_2$ in 0.1 M citrate buffer, with 20% (v/v) ethylene glycol at 25 °C at pH 5.41, 5.66, 5.88, 6.73, and 7.00. Aliquots were removed at the indicated times and assayed for PDC activity. First-order rate constants were extracted from the initial linear portions of the log (activity remaining) vs time plots.

Coupled Assay To Monitor Production of *p*-Methylcinnamaldehyde. Product **3** can be reduced by horse liver alcohol dehydrogenase (HLADH; Sigma, No. A-6128) with concomitant oxidation of NADH to NAD^+. In a typical assay mixture, 0.1 mM **1** was mixed with 35 units/mL of holo-PDC, 2 units/mL of HLADH, 0.23 mM NADH, and 1 mM TDP and $MgCl_2$ in 0.1 M citrate buffer, pH 6.0, with 20% (v/v) ethylene glycol at 25 °C in a total volume of 3 mL. The rate of disappearance of NADH at 360 nm (ϵ = 4250 M^{-1} cm^{-1}) was monitored. The rate of NADH oxidation in the absence of HLADH served as a control. When 1 mM compound **1** was used, 1.15 mM NADH was added and the rate of disappearance of NADH at 380 nm (ϵ = 1285 M^{-1} cm^{-1}) was monitored.

UV-Vis Determination of the Rate of Disappearance of Compound 1. The rate of disappearance of the starting material, compound **1**, could be conveniently monitored at 360 nm (ϵ = 2650 M^{-1} cm^{-1}) or 370 nm (ϵ = 1200 M^{-1} cm^{-1}) under the usual conditions of buffer, etc., in the absence or presence of 80 mM pyruvamide.

Isolation and Structural Characterization of the Second Product, Compound 2. To 1.0 L of 0.1 M citrate, pH 6.0, containing 0.1 mM TDP and $MgCl_2$ at 25 °C were added 1 mmol of **1** and 35 000 units of PDC. After 2 h of reaction, 390 g of ammonium sulfate was added to precipitate out the PDC and then the solution was centrifuged at 12 000 rpm for 20 min. Next, the supernatant was acidified to pH 1–2 by the addition of 2 M H_2SO_4. The solution was centrifuged at 12 000 rpm for 20 min and then extracted with ether (3 × 50 mL). The combined ether layers were dried ($MgSO_4$), and then the ether was removed at a rotary evaporator. The residue was applied to precoated TLC plates (silica gel GF, 20 × 20 cm, 1000 μm) in two different solvent systems: 1:2 methanol/chloroform and 1:7 methanol/chloroform. ^{1}H NMR (200 MHz, acetone-d_6/TMS): δ 7.30–6.80 (m, 4 H), 2.85 (t, 2 H, J = 7.5 Hz), 2.57 (t, 2 H, J = 7.5 Hz), 2.28 (s, 3 H). MS (m/z, relative intensity): 165 (M + 1, 2.7), 164 (25), 118 (21), 105 (100), 91 (18), 65 (10), 51 (8), 45 (10), 39 (13). Authentic dihydrocinnamic acid (Aldrich) in the same solvent and at the same field gave the following NMR data: δ 7.25 (m, 5 H), 2.92 (t, 2 H, J = 7.4 Hz), 2.61 (t, 2 H, J = 7.4 Hz).

Determination of Product Ratios by Proton NMR. In a total volume of 150 mL of 1 mM TDP and $MgCl_2$ in 0.1 M citrate buffer, pH 6.0, at 25 °C were added 1 mM compound **1** and 3060 units of PDC in the absence and presence of 80 mM pyruvamide. After 2 h of reaction, the solution was acidified to pH 1–2 by the addition of 2 M H_2SO_4 and then centrifuged at 12 000 rpm for 20 min. The supernatant was extracted with ether (3 × 50 mL), the combined ether layers were dried ($MgSO_4$), and the ether was removed at a rotary evaporator. The ratio of **3** to **2** was determined by proton NMR by comparing the area of the doublet at δ 9.6 of **3** to the area of the triplet at δ 2.57 corresponding to **2** in the presence and in the absence of pyruvamide.

Colorimetric Determination of the Accessible Cys on Holo-PDC. To 70 units of PDC dissolved in 1.0 mL of 0.1 M citrate buffer, pH 6.0, with 20% (v/v) ethylene glycol at 25 °C and containing 1 mM TDP, $MgCl_2$, and EDTA was added 5 mM compound **1** for 2 h, resulting in 7 units of activity remaining. A control experiment had all components except compound **1** and resulted in 70 units of activity remaining at the end of incubation. The concentration of protein was 14.6×10^{-5} and 14.7×10^{-5} M in the presence and absence of inhibitor, respectively, assuming a molecular weight of 250K for holo-PDC. Both protein samples were then inactivated by adding sodium dodecyl sulfate to a final concentration of 13%. Three-milliliter aliquots of these samples were then diluted to 10-mL final volumes with 15 mM phosphate, pH 8.5. These solutions were then titrated with 10-μL aliquots of 1.667 mM DTNB, and the absorbance at 412 nm (ϵ = 13 600 M^{-1} cm^{-1})[10a] due to the reduced form of DTNB 2-carboxy-4-nitrothiophenolate anion was recorded after each addition.

Stopped-Flow Experiments. These studies were conducted at 20 °C on a Hi-Tech Scientific PQ/SF-53 preparative quench-flow stopped-flow instrument. In a typical kinetic run, one syringe was loaded with 2 mM compound **1**, and 0.08 M pyruvamide in 0.1 M citrate, pH 6.0; the other syringe contained 70 units/mL of holo-PDC and 0.08 M pyruvamide in 0.1 M citrate, pH 6.0. The rate of formation of the enamine was monitored at 440 nm.

Attempted Enzymatic Interconversion of Compounds 3 and 2. To optimize the potential success of the experiment, we attempted to equilibrate **3** to a mixture of **2** and **3**, since in the absence of pyruvamide **2** predominates. To 140 mL of 0.2 M citrate, pH 6.0 and 6.8, containing

(8) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
(9) Schellenberger, A.; Hubner, G.; Lehmann, H. *Angew. Chem., Int. Ed. Engl.* **1968**, *11*, 886–887.
(10) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
(11) (a) Ullrich, J. *Ann. N. Y. Acad. Sci.* **1982**, *378*, 287–305. (b) Fluornoy, D. S.; Frey, P. A. *Biochemistry* **1989**, *28*, 9594–9602.

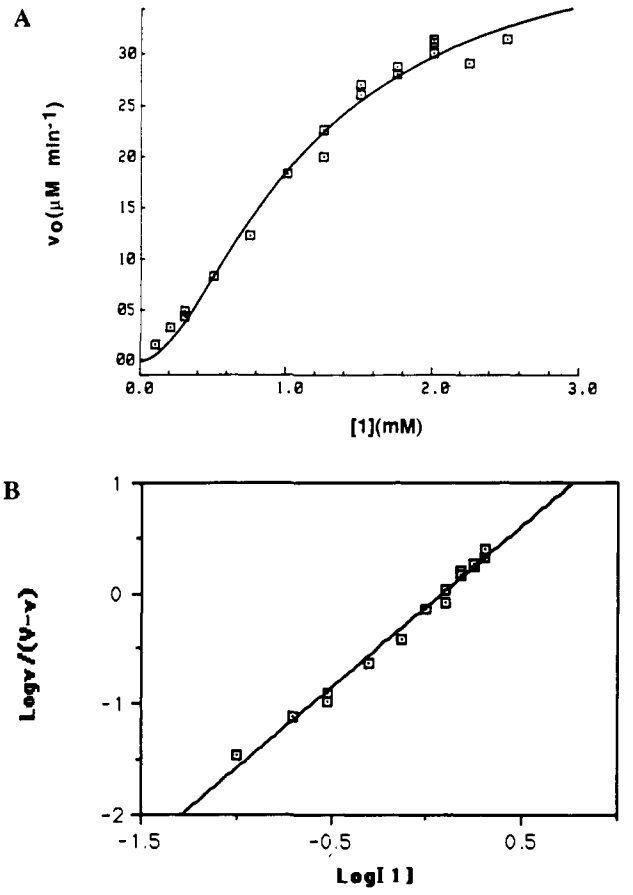


Figure 1. (A) Initial rate of PDC-catalyzed decarboxylation of **1**. The reaction contained 20 units/mL PDC, 0.1 M citrate buffer, pH 6.0, with 20% (v/v) ethylene glycol, and 1.0 mM TDP, $MgCl_2$, and EDTA and the indicated concentrations of **1** at 20 °C. The solid line represents the least-squares fit to the rate law $v_o = VS^2/[A + BS + S^2]$; the standard deviation is 1.35 and $V = 43.8 \pm 7$ μM/min. (B) Hill plot of PDC-catalyzed decarboxylation of **1**. Data are as in Figure 1A. The line drawn represents the least-squares line with $R^2 = 0.987$.

2.0 mM $MgCl_2$, EDTA, and TDP were added 4800 units of PDC and 0.099 g of cinnamaldehyde dissolved in 10 mL of MeCN, giving a 5 mM final concentration of cinnamaldehyde. After 50-h incubation at 4 °C, the solution was acidified to pH 1–2 by the addition of 2 M H_2SO_4 and then centrifuged at 12000 rpm for 20 min. The supernatant was extracted with ether (3 × 60 mL), the combined ether layers were dried ($MgSO_4$), and then the solution was concentrated to 60 mL at a rotary evaporator. Within detection limits GC–MS showed the presence only of the starting material, no dihydrocinnamic acid. It was also demonstrated that at the end of the incubation the PDC still retained ca. 25% of its initial activity.

Results

Assignment of the Absorbance Near 440 nm to the Enamine Structure. Treatment of compound **4** with the strong base $(TMS)_2NNa$ in Me_2SO yielded a new absorbance with maximum at 432 nm (ϵ = 10000 M^{-1} cm^{-1}). This absorption maximum is not present in the starting materials or products and clearly indicates the presence of conjugation between the thiazolium ring and the cinnamyl system, once there is an sp^2-hybridized carbon center connecting the two conjugated rings (see resonance structures in the second line of Scheme III).

Interaction of Compound 1 with Pyruvate Decarboxylase. Steady-State Kinetics. Earlier it was reported that at concentrations much lower than 1.0 mM compound **1** is a substrate.[6g] At larger concentrations, and in the presence of 20% ethylene glycol, compound **1** is also an inhibitor of PDC. The plot of the initial rate vs concentration for **1** is perceptibly sigmoidal, as can be seen in Figure 1A. The Hill plot (Figure 1B) provides a Hill coefficient of 1.46, indicating cooperativity. With respect to pyruvate, the Hill coefficient was found to be as high as 2.13.[13a]

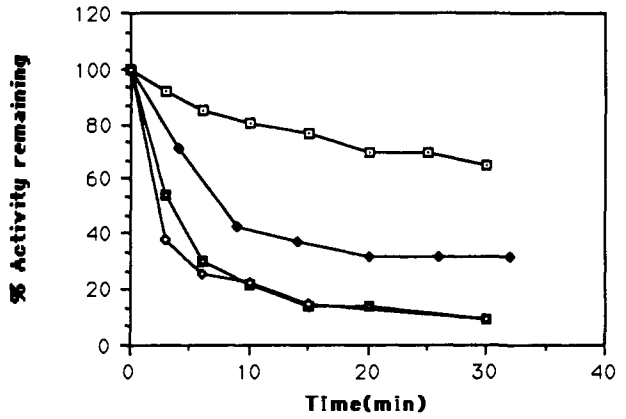


Figure 2. Time course of inactivation of PDC with compound **1**. Thirty-five units of PDC was incubated in 1 mL of 0.5, 1.0, 2.0, and 4.0 mM **1** and 1 mM TDP and $MgCl_2$ in 0.1 M citrate, pH 6.0, with 20% ethylene glycol at 25 °C. At the indicated times aliquots were removed for activity assay with the pH-stat.

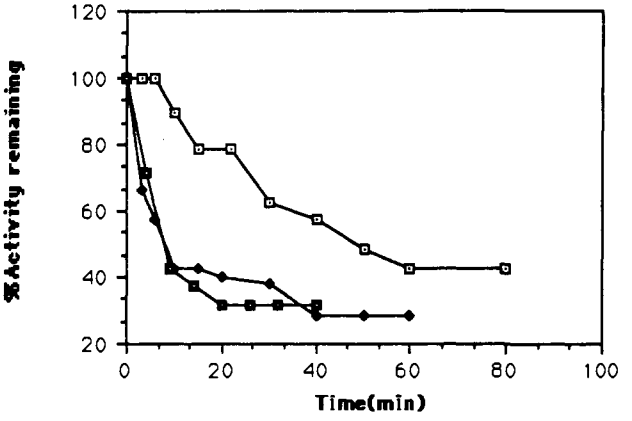


Figure 3. Effect of cofactors and pyruvamide on the time course of inactivation of PDC by compound **1**. The reaction mixture contained 35 units/mL of holo-PDC, 1 mM **1**, and 0.1 M citrate, pH 6.0, with 20% ethylene glycol at 25 °C without excess TDP and Mg^{+2} (open squares), with 1 mM TDP and Mg^{+2} (shaded squares), and with 0.08 M pyruvamide (diamonds). At the indicated times, aliquots were removed for pH-stat assay.

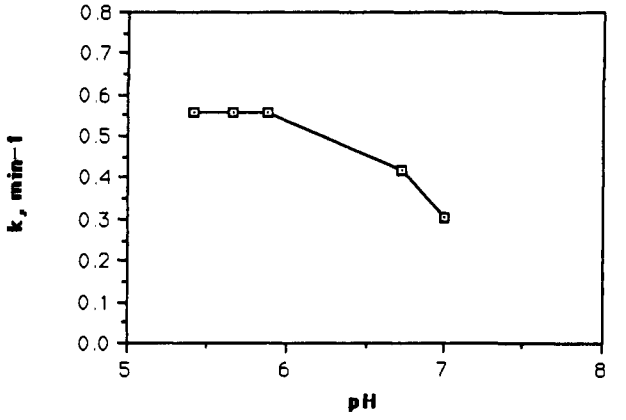


Figure 4. pH dependence of the first-order rate constant for inactivation of PDC by **1**. Fourty units of PDC was incubated with 5 mM **1** and 1 mM TDP and $MgCl_2$ in 0.1 M citrate with 20% ethylene glycol at 25 °C, at pH 5.41, 5.66, 5.88, 6.7, and 7.00. Aliquots were removed at appropriate time intervals and assayed for activity.

Inhibition of PDC by Compound 1. The time course of inactivation is shown in Figure 2. As is shown in Figure 3, addition of either excess cofactors or pyruvamide enhances the rate of inactivation. The rate constant for inactivation of PDC at a variety of pHs for a fixed high concentration of **1** is shown in Figure 4.

Material Balance on Turnover. There was some evidence over the past couple of years that the expected product of turnover,

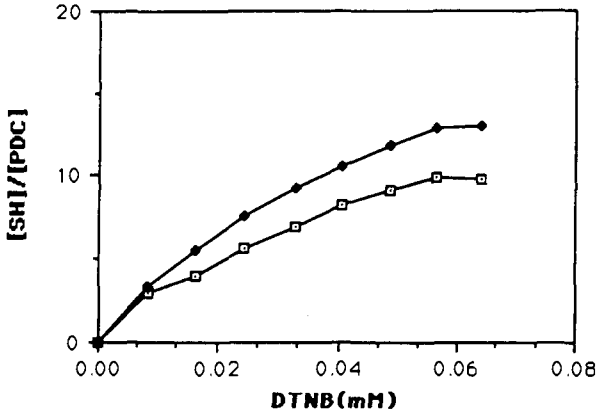


Figure 5. Titration of sulfhydryl groups on PDC using DTNB at 20 °C. PDC in the active form (upper curve) and inactivated (with compound **1**) form (lower curve) was titrated with DTNB as described in the Experimental Section. The absorbance at 412 nm, resulting from each addition of DTNB, was recorded and converted to concentrations that were plotted as a function of total DTNB concentration.

p-methylcinnamaldehyde (**3**), is not the only one. Suspicions were raised by the relatively small amount of aldehyde as detected by the repetitive-scan UV spectral measurements of the disappearance of the starting materials and concomitant product formation.[6g] In a systematic search for other than expected product (while quantifying the amount of **3** using the liver alcohol dehydrogenase coupled assay), the following reaction was set up. A 1 mM solution of **1** was incubated for 2 h at room temperature with 35 units/mL of holo-PDC in 0.1 M citrate, pH 6.0. At the end of this incubation the entire amount of **1** was nearly consumed, but only 0.25 mM **3** was detected according to the coupled assay. The major product was isolated as outlined in the Experimental Section. According to both ^{1}H NMR and MS, it is *p*-methyldihydrocinnamic acid (**2**).

Site of Inactivation on PDC. The Cys side chains of PDC were titrated with Ellman's reagent [5,5'-dithiobis(2-nitrobenzoate), DTNB] before and subsequent to inactivation with compound **1**, in a manner analogous to similar experiments outlined in ref 11. According to the colorimetric titration (Figure 5), active PDC contained 13.1 free SH per holoenzyme, compared to 9.9 for inactivated enzyme. Given the purity of the preparation, these numbers are in excellent accord with the four Cys/subunit (i.e., 16/tetrameric holoenzyme) recently reported for the genetic sequence.[12] The ratio of 13.1 to 16 suggests ca. 82% purity, with the 9.9 Cys remaining corrected for this 82% purity is consistent with modification of only a single Cys/subunit by **1**.

In a different experiment 525 units of PDC was incubated with 5 mM **1** in 10 mL of 0.1 M citrate, pH 6.0, with 20% ethylene glycol and containing 1 mM EDTA, TDP, and $MgCl_2$. After 2 h of incubation the activity diminished to 100 units. Next, the mostly inactivated PDC was dialyzed overnight against the same buffer but also containing 1 mM dithiothreitol at pH 6.0 and 6.8. A recovery of 10% activity at pH 6.0 and 15% at pH 6.8 was detected.

Kinetic Behavior of the Enzyme-Bound Enamine Derived from 1. In several previous reports the observation of a PDC/TDP-bound enamine intermediate derived from ring-substituted (*E*)-2-oxo-4-phenyl-3-butenoic acids was reported based on the observation of a new absorbance near 440 nm that was stable with time.[6b,c] In the previous cases, the enamines were observed from compounds that turned out to be potent inhibitors of PDC. When PDC was incubated with compound **1**, there again was observed the fomation of a new chromophore with absorption maximum near 440 nm (Figure 6).[14] Monitoring the A_{440} with time is instructive, showing a rapid increase, followed by a slower decrease (Figure 7). In Figure 8 it is demonstrated[14] that the addition of the regulator pyruvamide[13b] at 80 mM concentration to the

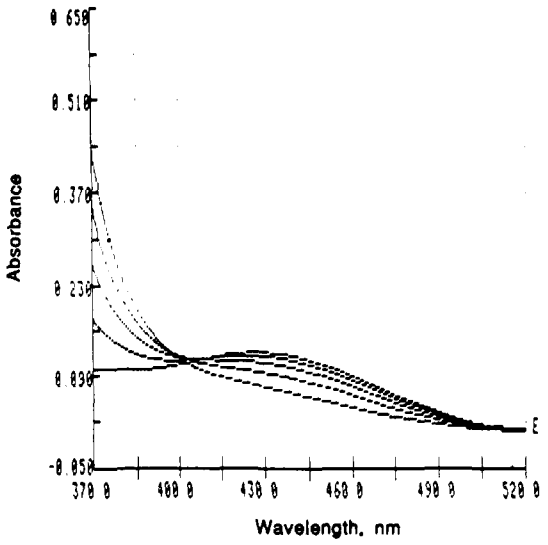


Figure 6. Repetitive-scan spectrum (Varian DMS 300, 2.0-nm spectral band width, 200 nm/min) between 370 and 520 nm of 3.0 mL of a solution containing 1 mM **1** and 35 units/mL holo-PDC in 0.1 M citrate, pH 6.0, with 20% ethylene glycol at 20 °C. The reference cell contained 0.5 mM **1** in 0.1 M citrate, pH 6.0, with 20% ethylene glycol but had no PDC.

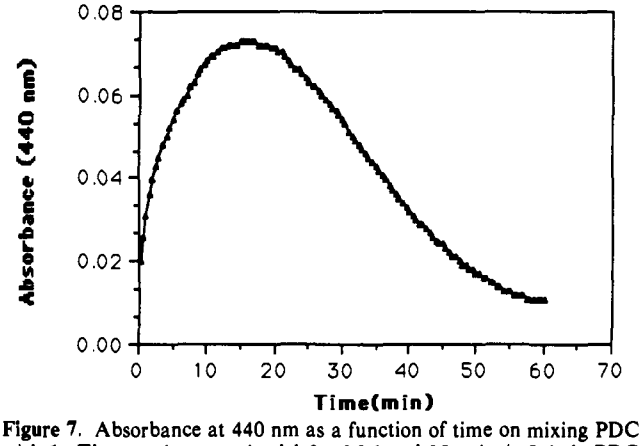


Figure 7. Absorbance at 440 nm as a function of time on mixing PDC with **1**. The reaction contained 1.0 mM **1** and 35 units/mL holo-PDC in 0.1 M citrate buffer, pH 6.0, with 20% ethylene glycol at 20 °C. The reference cell contained 0.5 mM **1** in 0.1 M citrate, pH 6.0, with 20% ethylene glycol but had no PDC.

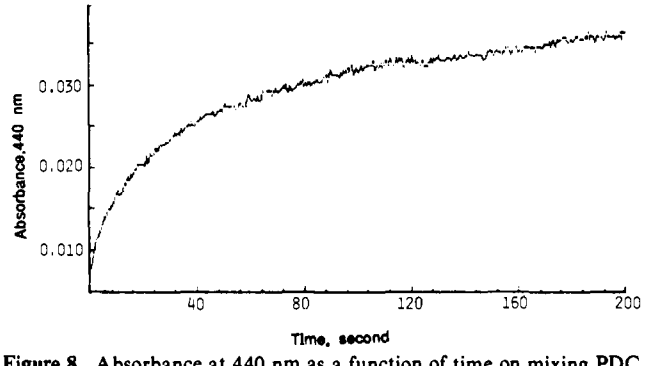


Figure 8. Absorbance at 440 nm as a function of time on mixing PDC with **1** in the presence of 80 mM pyruvamide (measured by stopped-flow at 20 °C; path length 1 cm). Conditions are as described in the Experimental Section.

reaction mixture of this allosteric enzyme[13a] accelerates the rate of enamine formation substantially. By the theory of consecutive reaction rates, data similar to those in Figure 7 were fitted to two

(12) Hohmann, S.; Cederberg, H. *Eur. J. Biochem.* **1990**, *188*, 615–621 and references therein.

(13) (a) Boiteux, A.; Hess, B. *FEBS Lett.* **1970**, *9*, 293–296. (b) Hubner, G.; Weidhase, R.; Schellenberger, A. *Eur. J. Biochem.* **1978**, *92*, 175–181.

Table I. Pseudo-First-Order Rate Constants for the Formation and Disappearance of the Enamine Intermediate Derived from Compound **1**

TDP and Mg^{2+}	pyruvamide, M	$k_1 \times 10^3$, s^{-1}	$k_2 \times 10^4$, s^{-1}
no excess		1.94 ± 0.09[a]	6.55 ± 0.52[a]
1 mM		6.94 ± 0.11[a]	5.01 ± 0.13[a]
no excess	0.08	97.6 ± 2.9[b]	

[a] The errors represent the standard deviation of the fit. [b] The error represents the standard deviation from the mean of four measurements.

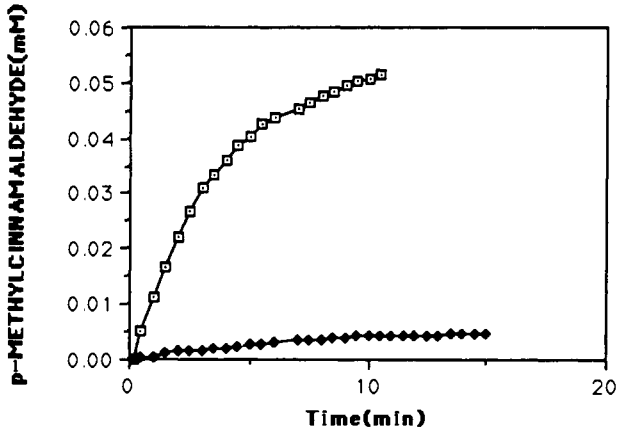


Figure 9. Rate of appearance of **3** with time in the presence (squares) and absence (diamonds) of pyruvamide measured with the coupled enzymes assay. The reaction contained 0.1 mM **1**, 35 units/mL holo-PDC, 2 units/mL HLADH, 0.23 mM NADH, and 0.1 M citrate buffer, pH 6.0, in the presence or absence of 0.08 M pyruvamide at 20 °C.

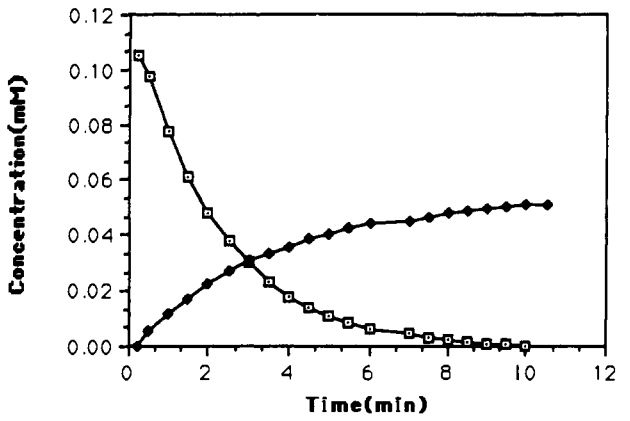


Figure 10. Rate of disappearance of **1** (squares) and formation of **3** (diamonds) catalyzed by PDC in the presence of pyruvamide. The solution contained 0.1 mM **1**, 0.08 M pyruvamide, and 35 units/mL holo-PDC in 0.1 M citrate, pH 6.0, with 20% ethylene glycol at 20 °C. Disappearance of **1** was monitored by the loss of absorbance at 360 nm. Appearance of **3** was monitored by the coupled assay by adding 2 units/mL HLADH and 0.23 mM NADH.

exponentials and gave the results listed in Table I.

Product Distribution in the Absence and Presence of the Allosteric Regulator Pyruvamide. The rate of formation of aldehyde product **3** was monitored in the absence and presence of 80 mM pyruvamide (Figure 9),[14] confirming that pyruvamide greatly accelerated the formation of the aldehyde product. In Figure 10 the rates of disappearance of starting material and appearance of aldehyde are shown in the presence of pyruvamide.[14] Figure 11 demonstrates clearly that the presence of 80 mM pyruvamide changes the product ratio significantly. In Parts A and B of Figure 12 repetitive-scan spectra demonstrate the same notion, enabling the direct comparison of the disappearance of starting material and appearance of the aldehyde in the absence and presence of the pyruvamide. The ratio of **3** to **2** was 1:3.1 in the absence of and 3:2 in the presence of 80 mM pyruvamide.

(14) Deleted in revision.

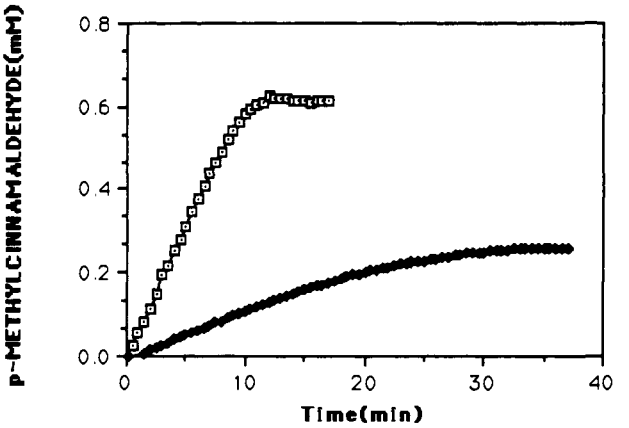


Figure 11. Rate of formation of **3** catalyzed by PDC in the presence (squares) and absence (diamonds) of pyruvamide. The solution contained 1.0 mM **1**, 35 units/mL of PDC, 2 units/mL HLADH, 1.15 mM NADH, 1.0 mM TDP, 1.0 mM $MgCl_2$, and 0.1 M citrate, pH 6.0, with 20% ethylene glycol in the presence or absence of 0.08 pyruvamide at 20 °C.

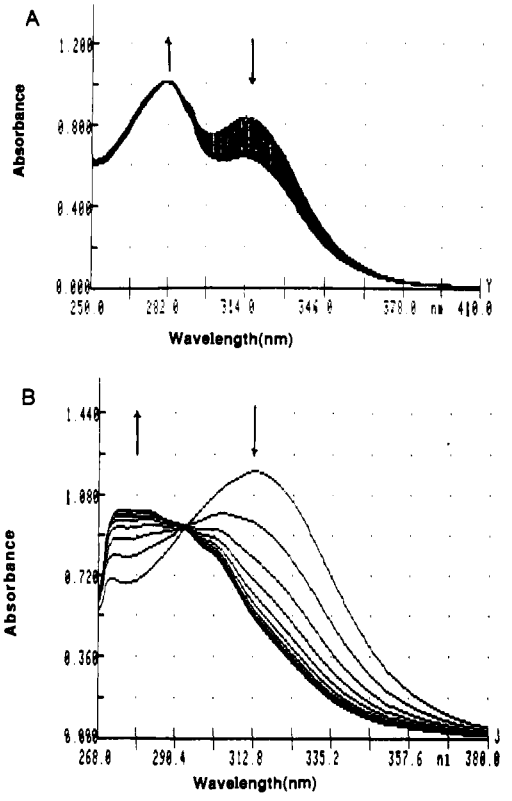
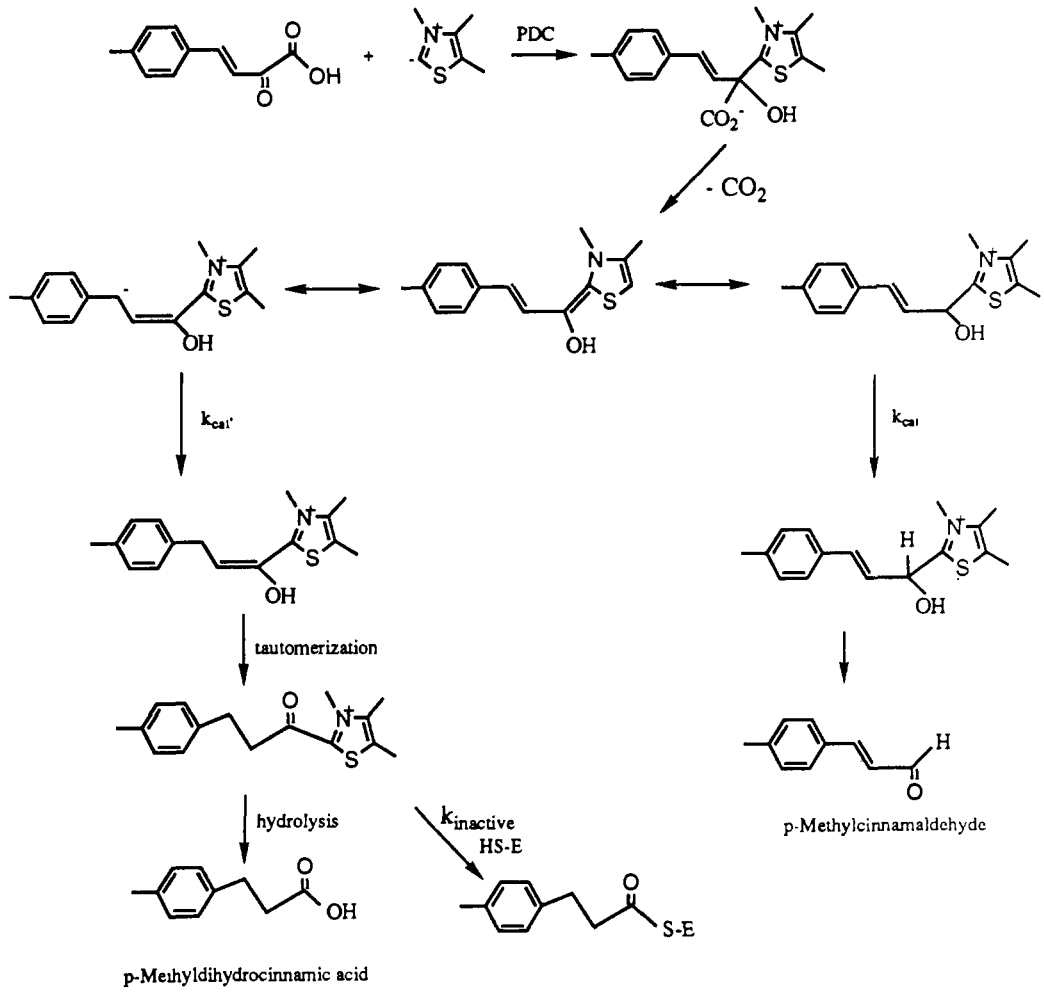


Figure 12. Repetitive-scan spectra (Varian DMS 300, 2.0 nm spectral band width, 200 nm/min) of compound **1**: (A) in the presence of PDC at 20 °C (reaction mixture contained 10 units/mL holo-PDC and 50 μM **1** in 0.1 M citrate, pH 6.0, with 20% ethylene glycol at 20 °C); (B) in the presence of PDC and pyruvamide at 20 °C (reaction mixture contained 10 units/mL holo-PDC, 0.08 M pyruvamide, and 50 μM **1** in 0.1 M citrate, pH 6.0, with 20% ethylene glycol at 20 °C). The arrows point to the direction of change with time.

Discussion

Unequivocal Identification of the Enamine Absorbance at 440 nm. First, it is worth emphasizing that by synthesis of **4** it could be demonstrated unequivocally that the absorbances generated with maximum near 430–440 nm from the variety of (*E*)-2-oxo-4-phenyl-3-butenoic acids[6b,c] do correspond to the enamine structure proposed in the first publication of this observation. This is important, since over the years there have been hypotheses

Scheme III



claiming that on PDC the enamine/2α-carbanion could not assume a planar structure here proposed (and also shown extensively in model compounds[5]). The highly conjugated enamine/2α-carbanion resonance hybrid is in evidence on the enzyme when derived from conjugated substrate analogues as large as, and larger than, **1**. Therefore, any hypothesis claiming steric hindrance to planarity of the enamine/2α-carbanion derived from the decarboxylation of smaller 2-keto acids, such as pyruvate, must be viewed with caution.

The data here support the contention that compound **1** is both a substrate and a time-dependent inactivator of PDC.

Substrate-like Turnover. The most novel feature of the current report is the finding that the enamine intermediate is very commonly misprotonated, i.e., leading to the dihydrocinnamic acid rather than to the expected cinnamaldehyde (Scheme II). Equally significant is the finding that the presence of pyruvamide, a known allosteric regulator discovered by the Schellenberger group,[13b] changes the regiospecificity of enamine protonation since the ratio of **3** to **2** changes from 25%/75% to 60%/40% on saturating the enzyme with 80 mM pyruvamide. Such a change in regiospecificity of protonation is best explained by a relative movement of the enamine with respect to protonating general acid (be it water or a general-acid catalyst on the enzyme) when the allosteric activator is added. It is as if the pyruvamide were correcting a misalignment in the protonation event. A further experiment (see the Experimental Section) showed that when cinnamaldehyde is added to a large concentration of PDC, it is not equilibrated to dihydrocinnamic acid. In other words, at least one of the steps required for such equilibration (formation of 2-(γ-hydroxycinnamyl)-TDP from PDC/TDP and cinnamaldehyde, its deprotonation at the cinnamyl γ-carbon, and then isomerization of the proton to the other allylic position) will not be favorable under the conditions of the experiment as designed.

Another aspect of the observations worthy of emphasis is the ability to monitor the fate of the enamine spectroscopically, i.e., with respect to both buildup and disappearance. The behavior shown in Figure 7 was analyzed according to the relationship

$$A_{440} = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \qquad (1)$$

Table I summarizes the rate constants for formation of enamine and its conversion to product. The following information is evident from the data. In the absence of excess cofactors (i.e., only the amounts always present during the purification procedure), the consumption of the intermediate is 3 times slower than the events culminating in decarboxylation. The presence of the typical large excess of the two cofactors (1 mM TDP and Mg^{2+}) affects the rate of enamine formation by a factor of ca. 3 but has little influence on its conversion to product(s). This specific rate enhancement of enamine formation by TDP has not been established before. The hysteretic behavior of PDC under the influence of TDP was reported by Sable and co-workers.[15] In addition, the rate of enamine formation is dramatically enhanced by the addition of 80 mM pyruvamide; under the latter conditions the rates could only be measured by stopped-flow techniques (the data in Figure 8 were fitted satisfactorily to a single exponential). The rate constant k_1 is enhanced ca. 50-fold compared to the case with no excess TDP or regulator. The transformation of enamine to product is also accelerated by pyruvamide, perhaps to a lesser extent (data not shown), but the complex kinetic behavior observed is as yet unexplained. In a recent paper on the kinetic fate of fluoropyruvate on PDC, it was suggested that allosteric effects are important only in product release.[16] The direct observation

(15) Egan, R. M.; Sable, H. Z. *J. Biol. Chem.* **1981**, *256*, 4877–4883.
(16) Gish, G.; Smyth, T.; Kluger, R. *J. Am. Chem. Soc.* **1988**, *110*, 6230–6234.

of the kinetic fate of the enamine intermediate derived from compound **1** suggests that both the rates of formation and conversion to product of the enamine are affected by the allosteric regulation.

Mechanism of Inactivation of PDC by Compounds Analogous to 1. Perhaps the most significant additional information is a direct demonstration that DTNB titration (affects only Cys side chains) of native and inactivated enzyme suggests that very close to one Cys/subunit was being inactivated. In addition to strongly implicating a Cys at the active center in the inactivation mechanism, the stoichiometry of interaction is just as striking for this mechanism-based inactivator and suggests similar inactivation mechanisms for all other related compounds as well.

Finally, how does the conjugated substrate analogue inactivate PDC at the Cys side chain near the active center? Scheme III summarizes some of the observations and includes a speculation.

The evidence is very strong that all of the analogues are decarboxylated (both from loss of labeled CO_2[6a,c] and from the detection of the cinnamaldehyde and dihydrocinnamic acid products). The pathway through the enamine then is clear. In the case of compound **1** the enamine is protonated at two sites that are allylic with respect to each other. Protonation at the cinnamyl γ-position (α to the thiazolium ring) leads to cinnamaldehyde, the normal product of enzymatic turnover. Protonation at the cinnamyl α-position (γ to the thiazolium ring) leads to the enol of 2-acyl-TDP; that on ketonization yields the 2-acyl-TDP. That species can undergo hydrolysis in aqueous milieu. Lienhard studied the decomposition of 2-acetyl-3,4-dimethylthiazolium ion in aqueous base and reported that the scission of the C2–C2α bond is preceded by hydration of the acyl group.[17] From Lienhard's data we estimate a rate constant of 2.3×10^{-3} s^{-1} for bond scission and 7.5×10^{-2} s^{-1} for hydration (the latter by a combination of pH-independent and pH-dependent pathways) of the keto function at pH 6.0 and 25 °C.[17] More recent data from Frey's laboratory on the hydrolysis of 2-acetyl-TDP at 24 °C leads to an estimate of 2.6×10^{-3} s^{-1} at pH 6.0.[18] The rate constant for product formation from Table I at 20 °C is ca. 6×10^{-4} s^{-1}, only 4-fold slower (at a slightly lower temperature than that used by Gruys et al.[18]). In other words, the hydrolysis of the 2-(*p*-methyldihydrocinnamoyl)-TDP to *p*-methyldihydrocinnamic acid may be partially rate limiting in product release, i.e., the pathway on the left side of Scheme III. However, these estimates from the work of others make it clear that chemical hydrolysis back to active TDP is very likely too fast to account for the long-term inactivation observed. The finding that one Cys/subunit is covelently modified concomitant with the inactivation requires a further modification of the schemes. The alternative pathway indicated in Scheme III suggests that the acyl group from 2-acyl-TDP may also (at least once in a while) be transferred to a nearby Cys on PDC, forming a thiol ester (2-acyl-TDP has a high group-transfer potential). Hydrolysis of the *p*-methyldihydrocinnamoyl thiol ester is slow, thereby inactivating the enzyme. A similar explanation was put forth by Frey and co-workers to account for the inactivation of pyruvate dehydrogenase with fluoropyruvate.[11b,19] While according to model studies in water 2-acetylthiazolium ion was found to be a poor acyl donor to thiol compounds,[17] Diago and Reed showed that in 98% dimethoxyethane/water significant amounts of acetyl group could be transferred from 2-acetylthiazolium ion to *n*-butyl mercaptan.[20] Therefore, on PDC such an acyl transfer from 2-acyl-TDP to a nearby Cys in an intramolecular reaction afforded by the enzyme would be clearly feasible, as was suggested by Frey and co-workers for the acetyl transfer from 2-acetyl-TDP (produced from fluoropyruvate) to a nearby Cys on pyruvate dehydrogenase.[11b] The rate of hydrolysis of thiol esters formed from alkyl mercaptans and alkanoic acids is very slow. For example, the second-order rate constants for alkaline and acid hydrolysis of methyl thiolacetate at 20 °C are 2.75 and ca. 0.2 $\times 10^{-3}$ M^{-1} min^{-1}, respectively, in 62% aqueous acetone, and the rates do not vary significantly with acetone content.[21] Accordingly, at pH 6.0 a half-life of *50 years* can be estimated in the absence of enzyme for such simple thiol esters. The pH-dependent inactivation profile (very similar in shape to that observed when PDC was inactivated with methyl methanethiosulfonate; Kuo, unpublished results) is consistent with higher hydrolytic rates for thiol esters at more alkaline pHs (i.e., faster recovery of activity). As mentioned under Results, the extent of reactivation of inactivated enzyme was slightly greater at pH 6.8 than at pH 6.

The results of this section provide the clearest evidence to date that there is a reactive Cys near the TDP locus in the tertiary structure of PDC, since there is no evidence for a Cys proximal to the putative TDP fold in the primary sequence of the enzyme.[22]

In conclusion, the complex fate of the enamine intermediate derived from **1** could be evaluated for the first time, providing details concerning the relative rates of both its formation and disappearance, the regiospecificity of its protonation, and the effects of the allosteric regulator on both rates and regiospecificity. It is important to emphasize that the technique used here is the clearest one to date that allows direct quantification of the rate enhancements produced by the allosteric activator pyruvamide. The model **4** when treated with a strong base provides an extinction coefficient for the enamine related to the conjugated 2-keto acids exploited by this group and also provides a useful methodology for active-site titrations of PDC with compound **1**, simply by quantifying the concentration of the enzyme-bound enamine.

Acknowledgment. We gratefully acknowledge financial support from the National Science Foundation (Grant DMB87-09758), the Rutgers University Busch Biomedical Fund, and Hoffmann-La Roche, Inc. Nutley, NJ, as well as analytical and MS services from Dr. F. J. Scheidl and Dr. W. Benz, respectively, from Hoffmann-La Roche. Finally, the continued generosity of the Anheuser Busch Brewing Co., Newark, NJ, in providing the live brewer's yeast is acknowledged.

Registry No. Cys, 52-90-4; TDP, 154-87-0; PDC, 9001-04-1; $(TMS)_2NNa$, 1070-89-9; Mg^{2+}, 7439-95-4; (*E*)-*p*-MeC_6H_4CH=CHCHO, 56578-35-9; *p*-$MeC_6H_4(CH_2)_2CO_2H$, 1505-50-6; 4,5-dimethylthiazole, 3581-91-7; *trans*-cinnamaldehyde, 14371-10-9; 2-(γ-hydroxy-(*E*)-cinnamyl)-4,5-dimethylthiazole, 133984-11-9; dihydropyran, 110-87-2; 2-[γ-[(2-tetrahydro-2*H*-pyran-2-yl)oxy]-(*E*)-cinnamyl]-4,5-dimethylthiazole, 133984-12-0; trimethyloxonium tetrafluoroborate, 420-37-1; 2-[γ-[(2-tetrahydro-2*H*-pyran-2-yl)oxy]-(*E*)-cinnamyl]-3,4,5-trimethylthiazolium tetrafluoroborate, 133984-14-2; pyruvamide, 631-66-3; (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid, 123333-23-3.

(17) Lienhard, G. E. *J. Am. Chem. Soc.* **1966**, *88*, 5642–5649.
(18) Gruys, K. J.; Datta, A.; Frey, P. A. *Biochemistry* **1989**, *28*, 9071–9080.
(19) Leung, L. S.; Frey, P. A. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 274–279.
(20) Daigo, K.; Reed, L. J. *J. Am. Chem. Soc.* **1962**, *84*, 659–662.
(21) Rylander, P. N.; Tarbell, D. S. *J. Am. Chem. Soc.* **1950**, *72*, 3021–3025.
(22) Hawkins, C. F.; Borges, A.; Perham, R. N. *FEBS Lett.* **1989**, *255*, 77–82.