obtained by following the rate of appearance of product **4** (ϵ = 7.07 × 10^3 M^{-1} cm^{-1} at 236 nm) using the nonequilibrium technique discussed elsewhere.[2] Stock solutions of **1** and **3** in ethanol were made up just prior to the start of the experiment. Detectable decompsition of these solutions was not observed during the lifetime of an experiment. Dilutions of 4-OT were made 2 h prior to the start of an experiment; otherwise inconsistent results were obtained.[2] No significant inhibition of the enzymes by ethanol was observed at ethanol concentrations below 2.5% (v/v). All results were reproducible in multiple runs. The kinetic data were fitted by nonlinear regression data analysis with Enzfitter (Elsevier Science Publishers, Amsterdam).

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Rate of Decarboxylation, Monitored via the Key Enzyme-Bound Enamine, of Conjugated α-Keto Acids by Pyruvamide Activated Pyruvate Decarboxylase Is Kinetically Competent with Turnover

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Abstract: The rate of formation of the covalent thiamin diphosphate-bound enamine/C2-α-carbanion intermediate monitored at 440 nm from conjugated mechanism-based inhibitors of the structure (*E*)-$YC_6H_4CH{=}CHCOCOOH$, where Y = *p*-Cl, *m*-NO_2, *m*-CF_3, was determined in the absence and presence of the allosteric activator pyruvamide on brewers' yeast pyruvate decarboxylase (PDC, E.C. 4.1.1.1). For all three compounds the first-order rate constant for enamine formation was accelerated from 15–150-fold by conversion of the enzyme to its activated form. The rate constant for enamine formation is 10^2–10^3 times faster than those estimated for inactivation. Comparing the k_{cat} (0.44 s^{-1}) to the rate constant for decarboxylation (0.653 s^{-1}) for Y = *p*-Cl leads one to conclude that enamine formation is kinetically competent to participate in the turnover pathway. Based on the maximum absorbance developed at 440 nm, and the ϵ = 10^4 at this wavelength for a model compound (Y = H), there appear to be four active sites per tetrameric holoenzyme. The k_{cat}/active site for pyruvate is estimated at ca. 40 s^{-1} at 20 °C, and the decarboxylation rate constant for pyruvate can be estimated to be 80 s^{-1}/active site at 20 °C, assuming decarboxylation and product release are equal in kinetic significance. The rate constants for decarboxylation by activated PDC for Y = *m*-NO_2, *m*-CF_3 (53 and 69 s^{-1}) are comparable to this estimated decarboxylation rate constant for pyruvate (80 s^{-1}). The *k*'s for Y = *m*-NO_2, *m*-CF_3 are also similar in magnitude to the decarboxylation rate constant (62–80 s^{-1} at 22 °C) reported for pyruvate oxidase, an enzyme with considerable sequence homology to PDC, and one that follows the same mechanism through decarboxylation.

Introduction

Quantitative aspects of substrate activation of brewers' yeast pyruvate decarboxylase (PDC, EC 4.1.1.1) were recently reported based on a variety of kinetic measurements.[1] It had also been demonstrated that pyruvamide, a nondecarboxylatable pyruvate surrogate is capable of fully activating PDC.[2] Evidence from this laboratory has been presented concerning the putative thiamin diphosphate (ThDP)-bound enamine/C2-α-carbanion intermediate that accumulates and is detectable on PDC (with a λ_{max} near 440 nm, Scheme I) when derived from conjugated α-keto acids with the basic structure of (*E*)-$YC_6H_4CH{=}CHCOCOOH$ (**I**), where Y is a *p*- or *m*-substituent.[3] Synthesis of an appropriate model compound for such enamines with identical absorption maximum gave support for the structure of the PDC-bound enamine intermediate.[4] Kinetic studies of the rate of formation of the enamine in the absence and presence of pyruvamide would provide direct evidence of enzyme activation and the PDC catalyzed decarboxylation process.

We report pre-steady-state kinetic results on the formation of the enamine intermediate on PDC from three conjugated substrate analogs (**Ia**, Y = *p*-Cl; **Ib**, Y = *m*-NO_2; **Ic**, Y = *m*-CF_3) in the absence and presence of saturating pyruvamide. These analogs are known to be mechanism-based inactivators (they are decarboxylated and also inhibit the enzyme),[5,6] and we hypothesized

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

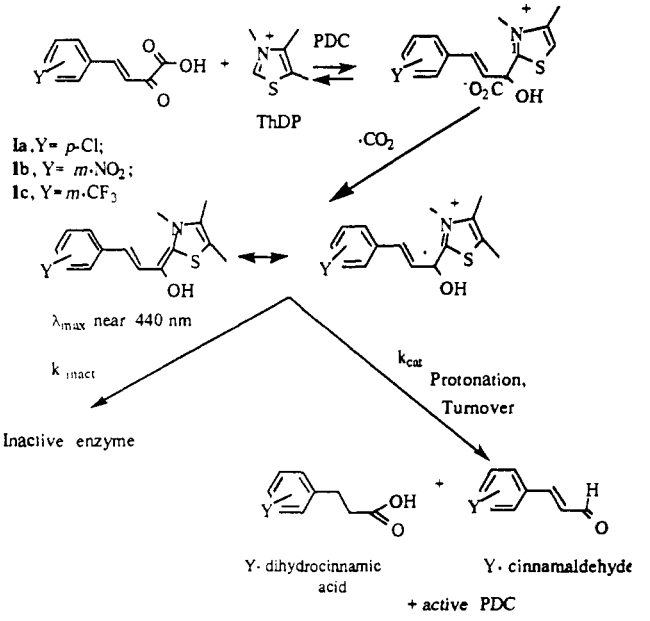

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Table I. Rate and Dissociation Constants Obtained for Enamine Formation from Conjugated Substrates at 20 °C[a]

substrate	k, s^{-1}	k', s^{-1}	K, M	K', M
Ia	0.65 ± 0.10		0.0042 ± 0.0010	
+ pyruvamide		9.8 ± 0.8		0.0015 ± 0.0002
Ib	2.1 ± 0.3		0.0052 ± 0.0010	
+ pyruvamide		53.2 ± 2.5		0.00075 ± 0.0007
Ic	0.43 ± 0.03		0.0096 ± 0.0007	
+ pyruvamide		68.9 ± 3.7		0.0019 ± 0.0002

[a] Typical reactions contained 0.2 mM ThDP, 0.2 mM $MgCl_2$, 0.1 mM Na_2EDTA, 0.1 M Na·citrate, pH 6.0, and 10% MeCN (to keep the substrate soluble), ca. 30 units/mL PDC in the absence and presence of 25 mM pyruvamide.

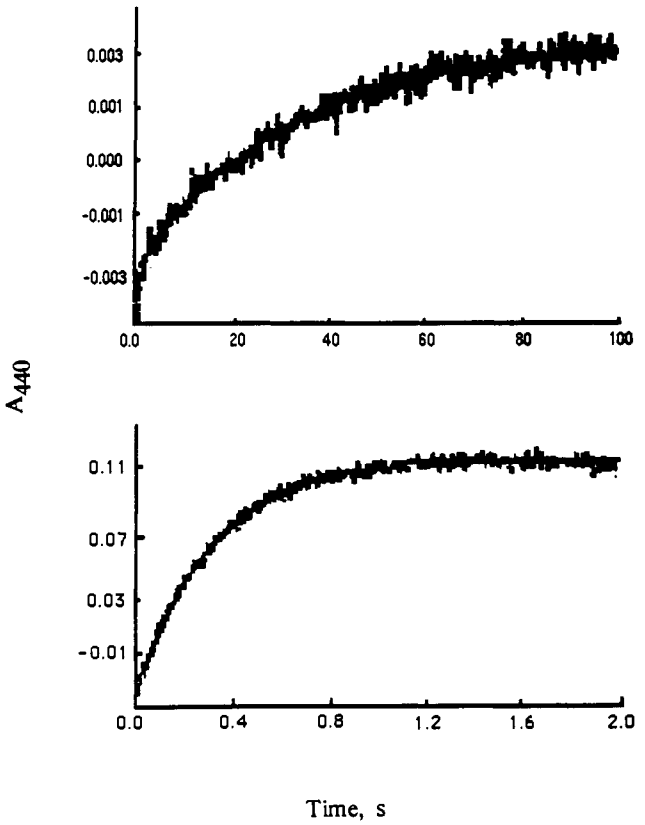

Figure 1. Rate of enamine formation from **Ic** and PDC in the absence (top, 0.7 mM **Ic**) and presence (bottom, 0.05 mM **Ic**) of 25 mM pyruvamide. Otherwise, conditions as in Table I.

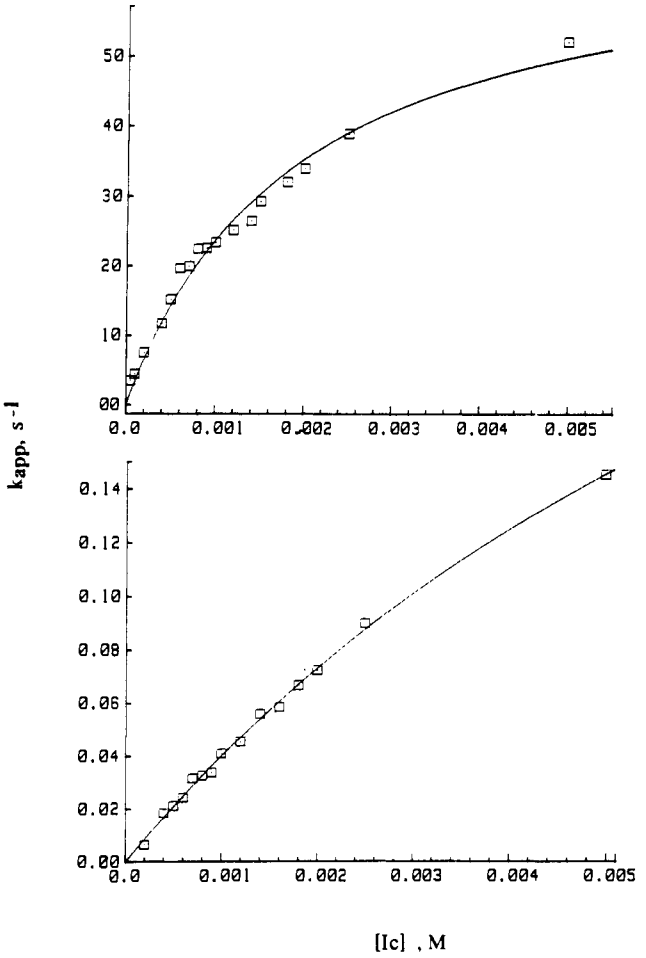

Figure 2. Apparent first-order rate constants (s^{-1}) for enamine formation (from plots such as in Figure 1) vs concentration of **Ic** in the absence (bottom) and presence (top) of 25 mM pyruvamide. Conditions as in Figure 1.

that the ultimate inactivation would have no influence on the pre-steady-state kinetics. The pre-steady-state results on enamine formation (as a monitor of the reactions culminating in decarboxylation) provide considerable new insight concerning the mechanism of PDC.

Experimental Section

Synthesis of Conjugated α-Keto Acids. Compounds **Ia**, **Ib**, and **Ic** were all synthesized by condensing the appropriately substituted benzaldehyde with pyruvic acid: compound **Ia** according to Datta and Daniels,[7] **Ib** according to Roushdi et al.,[8] and **Ic** according to Annan.[9] The purity and structure of each compound was confirmed by ^1H NMR and elemental analysis. The J_{vic} = 16 Hz across the vinyl double bound confirmed the *E* configuration in each compound.

Enzyme Purification and Assay. Initially, the homogeneous PDC from *Saccharomyces uvarum* was purified according to a procedure reported by Kuo et al.[10a] More recently, the purification protocol developed by Farrenkopf in this laboratory and capable of separating several pure homo- and heterotetrameric active fractions was employed.[10b] The α_4 isoform obtained in the highest purity (90+%) was used for this kinetic study. The enzyme activity was assayed using the pH-stat method.[11] One unit of activity is defined as the amount of PDC required to convert 1 μmol pyruvate to acetaldehyde per minute at 30 °C.

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 substrate analog in buffer at pH 6.0; the other syringe contained 60 units/mL of PDC in buffer at pH 6.0. The buffer used to dissolve the enzyme and the conjugated analogs was sonicated and degassed and always contained 0.1 M Na citrate, pH 6.0, with 0.2 mM ThDP, 0.2 mM $MgCl_2$, 0.1 mM EDTA, and 10% (v/v) CH_3CN. The rate of formation of the enamine was monitored at 440 nm. Control experiments were performed by mixing water alone, buffer alone, substrate analog in both syringes, and enzyme in both syringes. For experiments testing the effects of pyruvamide, the enzyme was preincubated with 50 mM pyruvamide

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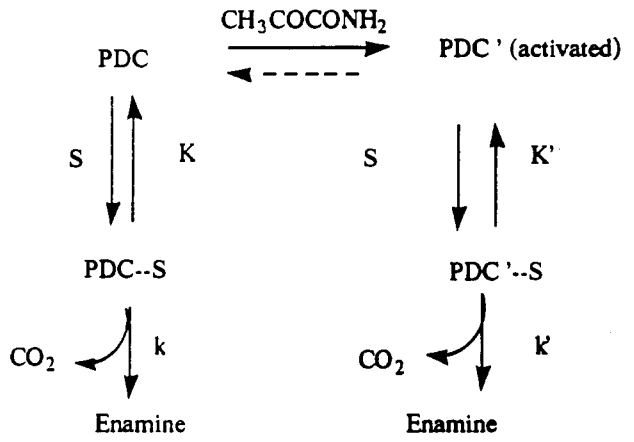
Scheme II. Proposed Mechanism for Enamine Formation



and then loaded in a syringe (resulting in 25 mM final pyruvamide concentration after mixing). The results were not altered by having 50 mM pyruvamide in each syringe.

Results and Discussion

As a representative of the experimental data, Figure 1 shows the formation of the enamine monitored at 440 nm for the reaction of PDC with **Ic** in the presence and absence of pyruvamide. Each transient in the presence or absence of pyruvamide was fit, using a nonlinear least-squares criterion, yielding first-order rate constants at each concentration of **Ic**. The resulting apparent rate constants for enamine formation vs concentration of **Ic** in the presence or absence of pyruvamide can be best fitted to a hyperbola (Figure 2). Table I summarizes the kinetic constants for the interaction of PDC with **Ia**, **Ib**, and **Ic** in the presence and absence of pyruvamide according to the mechanism in Scheme II. Parenthetically, we note that under the experimental conditions used ([**Ia**] ≫ [PDC]) the reate constants are independent of PDC concentration for the observation of the development of the PDC-bound intermediate:

$$k_{app} = k[I]/\{K + [I]\} \quad \text{and} \quad k'_{app} = k'[I]/\{K' + [I]\}$$

where I is the substrate/inhibitor, k and K are extracted from the hyperbolic fit and primed constants refer to pyruvamide-activated PDC.

Several conclusions can be drawn from the results of this kinetic study. (1) There is a dramatic effect of pyruvamide on the rate of enamine formation (decarboxylation), presumably due to the inability of **I** (unlike the natural substrate pyruvate) to activate PDC. Such differences between unactivated and activated PDC are difficult to monitor directly in the reaction with pyruvate. (2) The rate constant for enamine formation is 10^2–10^3 times faster than those estimated for inactivation.[5,6] Indeed, the effect of inhibition on enamine formation can be neglected. (3) The rate constant for inactivation by **Ia** is 0.0063 s^{-1} at pH 6.0, 30 °C, and inactivation results in ca. one of 70 turnover events, according to the release of $^{14}CO_2$ concomitant with the inactivation when [1-^{14}C]**Ia** was incubated with PDC at pH 6.0, 23 °C.[5] Therefore, k_{cat} for **Ia** is estimated as 0.44 s^{-1} or lower at pH 6.0, 20 °C. Since k for decarboxylation of this compound from Table I (0.653 s^{-1} at pH 6.0, 20 C) exceeds the estimated k_{cat}, one can conclude that enamine formation from this conjugated substrate is kinetically competent to participate in the turnover pathway. (4) Based on the maximum absorbance developed at 440 nm, and the ϵ of 10000 at this wavelength for a model compound (Y = H),[4] the "effective molecular weight" of PDC is ca. 70–85 kDa, and based on the known molecular weight of 240 kDa in a tetrameric structure,[10b,12] there appear to be four active sites per tetrameric holoenzyme. Therefore, the k_{cat}/subunit for pyruvate is 40 s^{-1} at 20 °C (calculated from the specific activity of 40 units/mg at 20 °C for pure PDC), and the decarboxylation rate constant for pyruvate can be estimated to be 80 s^{-1} at 20 °C, assuming decarboxylation and product release are equal in kinetic significance.[1,13] (5) The decarboxylation rate constants for **Ib** and **Ic** (53 and 69 s^{-1}) are similar to the estimated rate constant of decarboxylation for pyruvate (80 s^{-1}), and the K's are also similar to that estimated for pyruvate (0.001 46 M).[1] It appears that the rate constants for decarboxylation of conjugated substrate analogs and pyruvate by activated PDC are comparable. (6) The decarboxylation is ca. 10^6 times faster than in the best aqueous model system to date.[14]

It is also noteworthy that the decarboxylation rate constants for **Ib** and **Ic** are similar in magnitude to the decarboxylation rate constant (62–80 s^{-1} at 22°C) reported for pyruvate oxidase, an enzyme with considerable sequence homology to PDC, whose first function is decarboxylation (enamine formation), totally analogous to the reaction here monitored.[15] The mechanism of ThDP-dependent enzyme catalyzed decarboxylation of pyruvate is known to proceed by attack of the ThDP C2 carbanion on the α-keto group of pyruvate to form 2-(1-carboxy-1-hydroxyethyl)ThDP.[16] Studies on the decarboxylation of the model compound 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride[17] revealed a striking catalytic effect of desolvation: the rate of decarboxylation in ethanol was 10^4-10^5-fold faster than in water. According to protein sequence alignments, for all ThDP-dependent enzymes there is a conserved ThDP sequence motif.[18] For several of those performing decarboxylation (acetolactate synthase, pyruvate oxidase, pyruvate decarboxylase,[19] and benzoylformic acid decarboxylase[20]) there is also considerable sequence homology. It is tempting to speculate that the 60–100 s^{-1} rate constant around 20 °C is the maximum value of the unimolecular decarboxylation rate constant that can be achieved by ThDP-dependent decarboxylases, presumably by optimization of the protein structure surrounding the ThDP-bound α-keto acid.

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