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CONJUGATED α-KETO ACIDS AS MECHANISM-BASED INACTIVATORS OF BREWER'S YEAST PYRUVATE DECARBOXYLASE: ELECTRONIC EFFECTS OF SUBSTITUENTS AND DETECTION OF A LONG-LIVED INTERMEDIATE

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A series of phenyl substituted *E*-4-phenyl-2-keto-3-butenoic acid derivatives were synthesized (*p*-Cl, *m*-Cl, *p*-NO_2, *m*-NO_2, *o*-NO_2, 3,4-Cl_2, 2,6-Cl_2, *p*-CH_3O, *p*-$(CH_3)_2N$) and tested as potential irreversible inhibitors of brewer's yeast pyruvate decarboxylase (EC 4.1.1.1). All those derivatives with electron withdrawing substituents were found to be time-dependent inactivators of the enzyme, unlike the *p*-CH_3O- and *p*-$(CH_3)_2N$ derivatives. Detailed kinetic studies with the *m*-nitro derivative (the most potent inhibitor) indicated that this compound formed reversible complexes with the enzyme at two sites (supposed regulatory and catalytic with K_i values of 0.026 and 0.13 mM, respectively) prior to irreversible inactivation of the enzyme. In addition, concurrently with the inactivation, addition of the *m*-NO_2 derivative to the enzyme produced a new VIS absorbance with λ_{max} near 430 nm. This absorbance was attributed to the enzyme-bound enamine intermediate. The time course of formation and disappearance of the intermediate could be determined and provided detailed information about the mechanism of the enzyme.

KEY WORDS: Yeast pyruvate decarboxylase, α-keto acids, mechanism-based inactivator, enamine.

INTRODUCTION

An important concept in the design of enzyme inhibitors involves slowing down the rate of the product release step for example by stabilizing putative intermediates on the enzyme surface. Such inhibition may have two mechanistically informative consequences: (a) formation of a covalent bond between the inhibitor and a nucleophile of the protein, and (b) possible observation of the intermediate by spectroscopic or kinetic means.

We recently reported a novel inhibitor of this type for brewer's yeast pyruvate decarboxylase (E.C. 4.1.1.1) or PDC, an enzyme that requires thiamin diphosphate (TDP) and Mg^{2+} for its activity, and performs the reaction depicted below:

$$CH_3COCO_2H + PDC \rightarrow CH_3CHO + CO_2 \quad (1)$$

The conjugated substrate analog *E*-4-(4-chlorophenyl)-2-keto-3-butenoic acid, *p*-CPB led to irreversible inactivation of PDC[1]. Also [1-^{14}C]CPB lost radioactive CO_2

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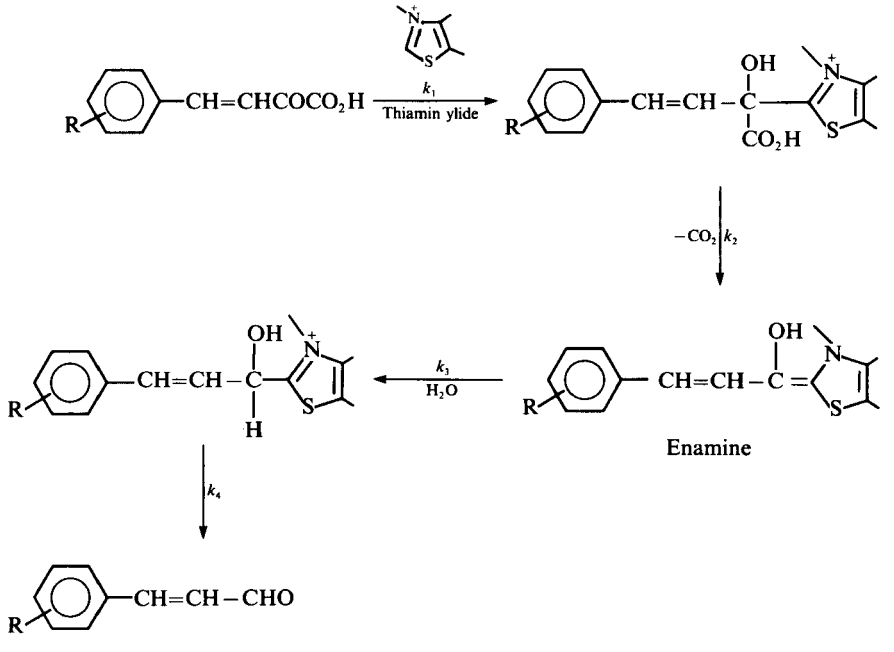

SCHEME 1

concurrently with the inactivation of the enzyme. The ratio of CPB turned over by PDC to CPB participating in inactivation was about 70 at pH 6, the pH optimum of the enzyme[1]. In addition, also concurrently with the inactivation there developed a new chromophore with absorbance maximum at 440 nm, that was attributed to the enzyme-bound enamine intermediate[2] shown in Scheme 1. A reasonable explanation of these findings is that the enamine intermediate derived from CPB has a long enough half life (by virtue of its highly resonance stabilized structure) to allow it to react with an enzymic nucleophile and to enable its spectroscopic observation.

In this communication we present data on the inactivation of PDC by a number of other compounds resembling *p*-CPB, i.e. bearing a variety of substituents on the phenyl ring. For the *m*-nitro derivative we also present the time course of the fate of the enamine intermediate. Very detailed mechanistic information could be deduced from the observations.

MATERIALS AND METHODS

Synthesis of conjugated α-keto acids. The compounds listed in Table I were all synthesized by condensing the appropriately substituted benzaldehyde with pyruvic acid: the compound *p*-CPB according to Datta and Daniels[3], *p*-DPB according to Kageura *et al.*[4] and all others according to Roushdi *et al.*[5]. The purity and structure of each compound was confirmed by ^{1}H n.m.r. The J_{vic} = 16 Hz across the vinyl double bond confirmed the *E* configuration in each.

TABLE I
Inactivation of brewer's yeast pyruvate decarboxylase by aryl substituted 4-phenyl-2-keto-3-butenoic acids at 30° C, pH 6.0

R	Name	Inactivation half life, min for the inhibitor concentration indicated	
		1.0 mM	0.5 mM
p-Cl	*p*-CPB	3.50	4.90
m-Cl	*m*-CPB	2.64	3.58
3,4-Cl	3,4-CPB	2.25	3.40
2,6-Cl	2,6-CPB	0.68	1.25
p-NO_2	*p*-NPB	2.78	4.13
m-NO_2	*m*-NPB	2.06	2.30
o-NO_2	*o*-NPB	0.97	1.56
p-CH_3O	*p*-MPB	—	—
p-$(CH_3)_2N$	*p*-DPB	—	—

Enzyme purification and assay. PDC from brewer's yeast was purified according to a protocol previously described.[1] The enzyme was assayed by the pH-stat method.[6,7] Inhibition kinetics were performed at 30.0 ± 0.1° C. A typical incubation mixture contained 15 units of PDC in 0.5 ml also containing 0.2 mM TDP, 0.2 mM $MgSO_4$, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride in 5% (v/v) ethylene glycol and 0.1 M citrate, pH 6.0 at 30° C. At various time intervals 30 μl aliquots were removed from the incubation mixture and assayed for PDC activity at 30° C by the pH-stat assay.

UV-VIS spectroscopic studies were performed on a Cary 219 instrument equipped with a thermostatted cell compartment.

RESULTS AND DISCUSSION

Inactivation Profiles by Substituted 4-Phenyl-2-keto-3-butenoic Acids

Table I presents the relevant data. The $t_{1/2}$ values for inactivation were estimated from the initial linear portions of the log (activity remaining) vs. time profiles. Several qualitative features of the data are apparent. Firstly, electron withdrawing substituents enhance the rate of inactivation. Within one half hour incubation time no evidence could be found for time-dependent inactivation by the *p*-methoxy- or the *p*-dimethylamino compounds. The inductive effect of the electron withdrawing substituents appears to be the dominant factor in the inactivation process: compare *p*-chloro to *m*-chloro or *p*-nitro to *m*-nitro. The time courses of inactivation are presented for the *m*-nitro, *o*-nitro and 2,6-dichloro derivatives. While the *m*-nitro (as well as the *p*-nitro, *p*-chloro, *m*-chloro, 3,4-dichloro) derivative leads to monotonic inactivation (Figure 1), the two ortho substituted examples, the *o*-nitro and 2,6-dichloro compounds, do not (Figures 2 and 3, respectively). The latter two compounds lead to rather rapid inactivation ($t_{1/2}$ value less than one minute). However, at a later time some of the activity is recovered. In accord with out previous data[1,2], we interpret these findings invoking Scheme I. The closer the electron withdrawing substituent is located to the reaction center the faster is formed the enamine, i.e.,

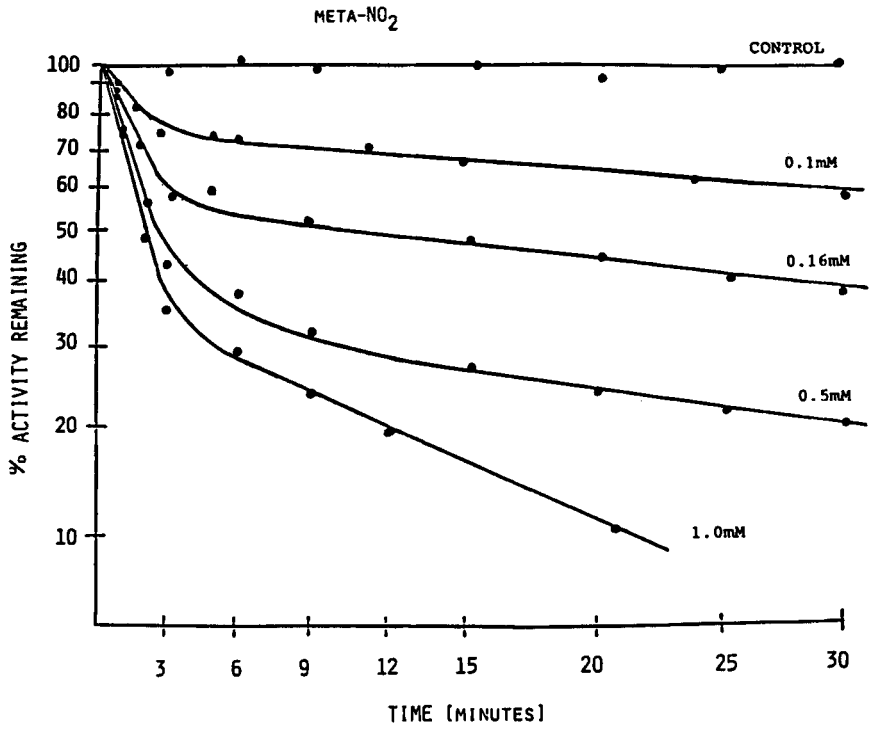

FIGURE 1 Time course of inactivation of PDC by *m*-NPB at pH 6.0 and 30°C at the indicated concentrations of inhibitor. See experimental conditions under Materials and Methods.

electron withdrawal makes the carbonyl carbon more susceptible to nucleophilic attack (k_1) by the thiamin ylid. This is followed by faster decarboxylation (k_2) leading to the enamine[8]. The enamine can next be protonated (leading to turnover via k_3 and k_4), or can give the undesirable side reaction, probably upon reaction with an enzymic nucleophile and leading to inactivation of the enzyme. Apparently, in the case of the ortho substituted derivatives (Figures 2 and 3) the enamine, while formed rapidly, can be released by protonation more readily than that from the *m*-nitro derivative (or indeed from the other compounds in Table I). This leads to regeneration of some of the activity. Since presumably the substrate is depleted more rapidly for the ortho substituted analogs, the inhibition comes to a halt (note behavior after 15–20 min). A possible reason for the unusual behavior of the ortho substituted derivatives is steric inhibition to resonance stabilization of the enamines derived from these compounds. The *m*- and *p*-substituted compounds possess an uninterrupted conjugated system between the aromatic phenyl ring and the side chain π-electronic structure, hence these compounds would be converted to more highly resonance stabilized enamine intermediates than the ortho derivatives.

Figure 4 demonstrates the detailed kinetic analysis of inhibition by the *m*-nitro derivative according to a Kitz and Wilson[9] double reciprocal plot. According to this analysis the double reciprocal plot is linear at high inhibitor concentrations when plotting against reciprocal of the inhibitor concentrations, and linear at low inhibitor concentrations when plotting against the reciprocal of the square of the inhibitor

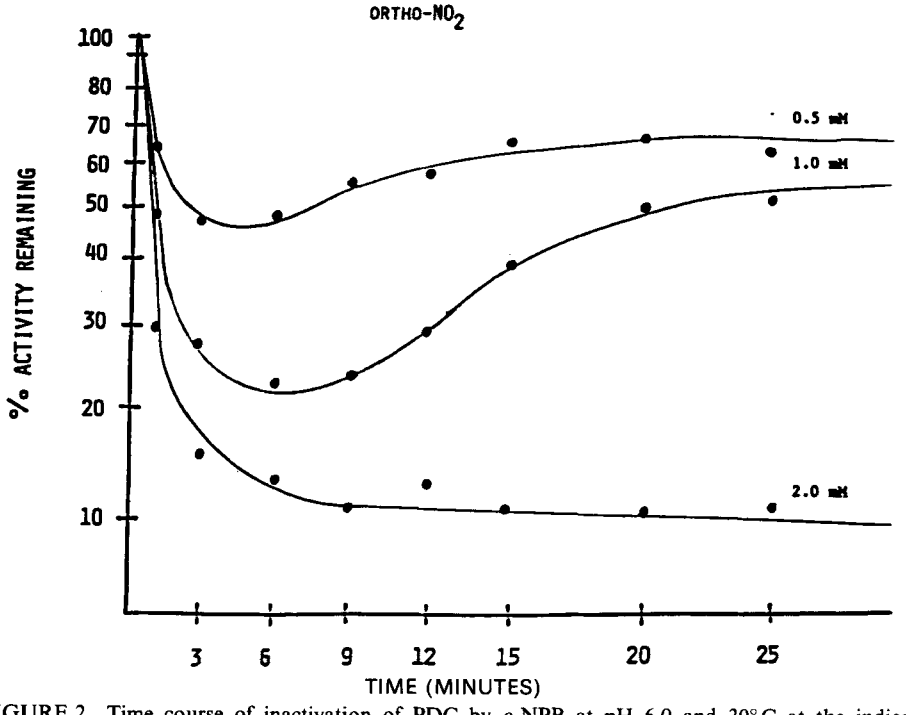

FIGURE 2 Time course of inactivation of PDC by *o*-NPB at pH 6.0 and 30°C at the indicated concentrations of inhibitor. See experimental conditions under Materials and Methods.

concentration. We interpret these results in terms of a two-site binding mechanism: just as the substrate pyruvate[10], *m*-NPB also binds to both the regulatory and the catalytic center (as was found for *p*-CPB earlier[1]) reversibly prior to irreversible inactivation, presumably at the catalytic site. From Figure 4 one can deduce K_{ir} (regulatory) = 0.026 mM (K_{ir} for *p*-CPB is 0.3 mM) K_{ic} (catalytic) = 0.13 mM (K_{ir} for *p*-CPB is 0.7 mM) compared to K_m's of 0.8 and 2.0 mM for pyruvate[10]. The *m*-NPB compound gave a $k_{inactivation}$ (the rate constant for inactivation that would result at infinite inhibitor concentration) of 0.33 min^{-1} compared to 0.38 min^{-1} for *p*-CPB[1]. Thus while the rate constants for inactivation were similar, the *K* values were significantly different. Especially noteworthy are the 20–30 fold decreases in K_i values compared to K_m values (admittedly the latter need not represent true $K_{dissociation}$). It is important to emphasize that our earlier reports only dealt with *p*-CPB[1,2] and the present results underline the generality of those observations. In particular, the case for two-site binding observed for all α-keto acids tested to date, is strongly supportive of a regulatory mechanism for this enzyme.

Time Resolution of the Fate of the Enzyme-Bound Enamine Intermediate

According to Table 1, *m*-NPB was the most potent inhibitor of PDC among those that did not lead to partial regeneration of active enzyme. Therefore, this compound was also used in VIS spectroscopic studies attempting to detect the enzyme-bound

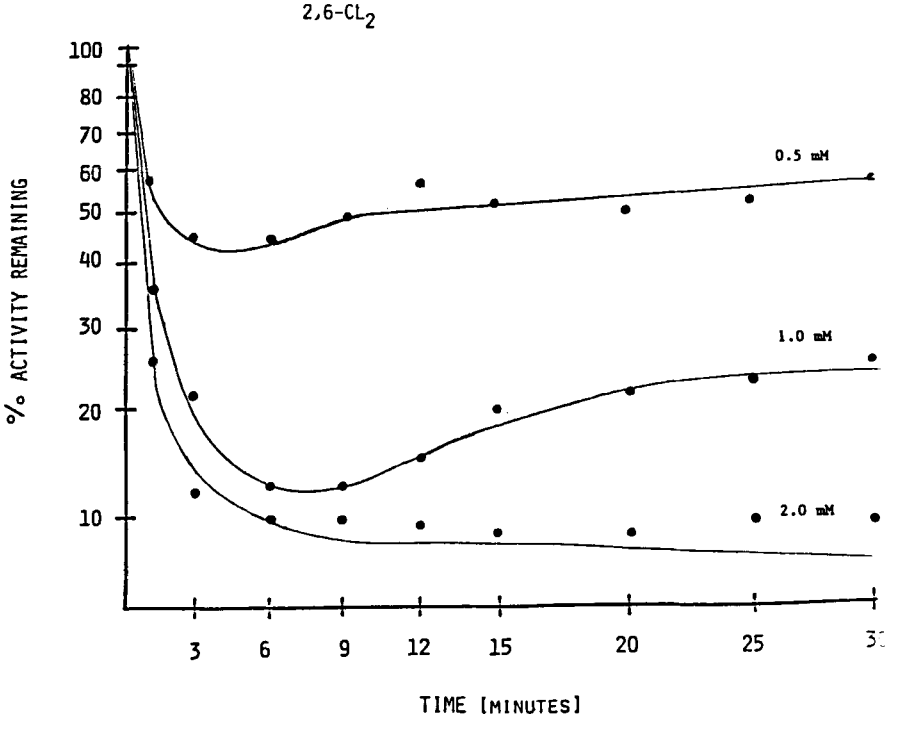

FIGURE 3 Time course of inactivation of PDC by 2,6-CPB at pH 6.0 and 30° C at the indicated concentrations of inhibitor. See experimental conditions under Materials and Methods.

enamine intermediate. Figure 5 shows a repetitive scan VIS spectrum that results from mixing *m*-NPB with PDC · TDP at 10° C. A new absorbance developed with λ_{max} near 430 nm, an observation very similar to that made for *p*-CPB.[2] The maximum absorbance attained was measured at a variety of enzyme concentrations (it was found to be proportional to the total enzyme concentration present) and provided an estimate of $\varepsilon_{440} = 4 \times 10^3$ (calculated for a subunit molecular weight of 60,000). We attribute this value to the enamine structure in Scheme I (derived from *m*-NPB). The pH dependence of the rate of formation of the A_{440} was determined next from the approximately first-order initial buildup of the absorbance at 10° C. The apparent first-order rate constants when plotted against pH describe a bell-shaped profile with a pH optimum near 6 (Figure 6). The shape and pH optimum of this curve are totally analogous to those obtained for the overall enzymatic reaction.[11] In the very least, one can conclude that the same amino acid side chains (with the same p*K*'s) govern both the rate of the overall enzymatic reaction and the formation of the enamine. Very likely, the same rate-determining step is observed in both rate-pH profiles.

A typical plot of the build-up of the A_{440}, i.e., the enamine, in water is shown in Figure 7. Such experiments were performed at a number of pH values at 5, 10 and 15° C. Due to the very small absorbance changes observed (much less than 0.1) rather large instrumental noise resulted. Nevertheless some qualitative conclusions are

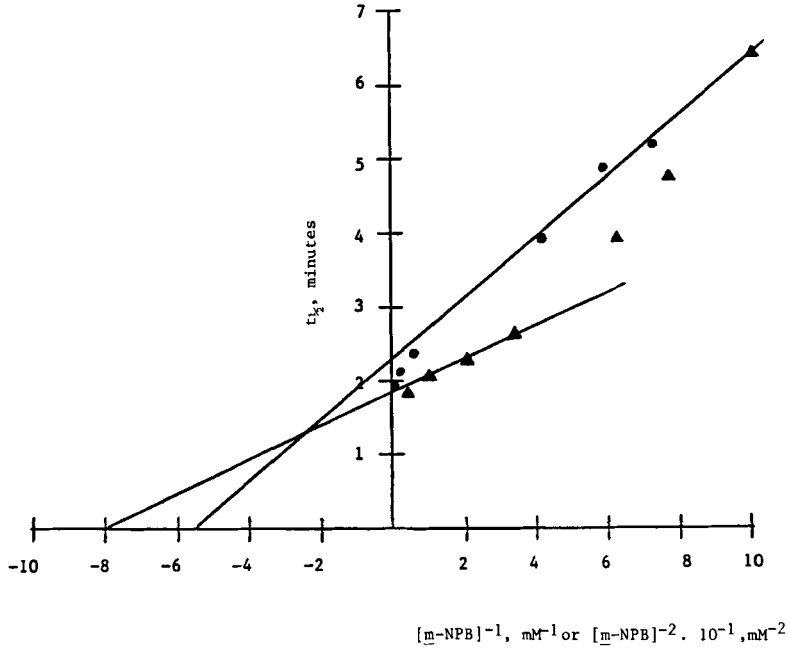

FIGURE 4 Double reciprocal plot for the inactivation of PDC by *m*-NPB at 30° C, pH 6.0. The half-lives for inactivation were obtained from the initial linear portions of curves such as those in Figure 1. The plots were constructed according to Kitz and Wilson[9] plotting $t_{1/2}$ against [*m*-NPB]$^{-1}$ (▼) and [*m*-NPB]$^{-2}$ (●), respectively.

already evident. The most significant of these is that both the formation and consumption of the intermediate can be monitored. Furthermore, several experimental conditions were found under which the formation of A_{440} followed distinctly biphasic kinetics. We tentatively interpret the biphasic build-up of A_{440} analogously to that observed in the hydrolysis of *p*-nitrophenyl esters by chymotrypsin and other serine proteases[12,13]: rapid build-up of an enzyme-bound intermediate, followed by slower, rate-limiting turnover. Applied to Scheme I, this implies that under most conditions observed, the interaction of PDC · TDP with *m*-NPB leads to rapid formation of the enamine that is followed by slower, rate limiting protonation and release from the enzyme. The ultimate diminution of A_{440} very likely corresponds to conversion of the chromophore to a covalently-bound species that inactivates the PDC · TDP complex vis-á-vis further substrate turnover. It is significant to note that in a parallel pH-stat study of the activity of the enzyme remaining with time vis-á-vis pyruvate turnover, no activity returned at the time when the A_{440} was observed to diminish. Therefore, the decreasing magnitude of A_{440} is not simply due to consumption of *m*-NPB. To test the above speculation concerning the interpretation of the biphasic nature of the buildup of A_{440}, we performed solvent deuterium isotope effect studies. The slower phase should be more susceptible to such effects, if C-protonation (k_3 in Scheme I) is kinetically significant. Initial studies indicated that the pH optimum of the build-up of A_{440} shifted about 0.5 pK units toward the alkaline region in D_2O (not unexpected). Figure 8 demonstrates an experiment similar to that in Figure 7 but at pH 6.49_{app} in

FIGURE 5 Repetitive scan spectrum between 363 and 550 nm (from left to right) that results on addition to a mixture of 0.7 ml "solvent" (contains 0.1 M pyrophosphate, 1 mM TDP, 1 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM phenylmethane sulfonyl fluoride) and 0.2 ml of 10 mM *m*-NPB (in both reference and sample cuvettes), 200 μl (160 units/ml or about 30 units total) PDC in the sample cuvette only at 10°C. The spectra were recorded at 0.1 absorbance range, 3 nm band width, 2 nm/s and 10 nm/cm in a 1 ml 10 mm path cuvette. The maximum is near 430 nm.

D_2O at 15°C (all solution pH values in D_2O were measured at a glass electrode and are uncorrected). A comparison of the relative rates of the three phases is reasonable since both pH 5.96 in H_2O (Figure 7) and pH 6.49 in D_2O (Figure 8) represent pH values very near the pH optimum of the enzymatic reaction. The second phase is subject to a much larger solvent isotope effect (8–9) than the first (2–3) and third (3–4). These observations give us confidence that the second kinetic phase indeed represents protonation of the enamine.

The spectral observations can be summarized as follows:

$$\text{PDC} \cdot \text{TDP} + m\text{-NPB} \xrightarrow[k_1,k_2]{\text{Phase 1}} \text{Enamine} \xrightarrow[k_3\,(\text{C-protonation})]{\text{Phase 2}} \alpha\text{-hydroxyalkyl TDP} \xrightarrow[k_4]{} \text{product}$$

$$\text{Enamine} \xrightarrow{\text{Phase 3}} \text{Inactive-enzyme}$$

enamine chromophore is destroyed

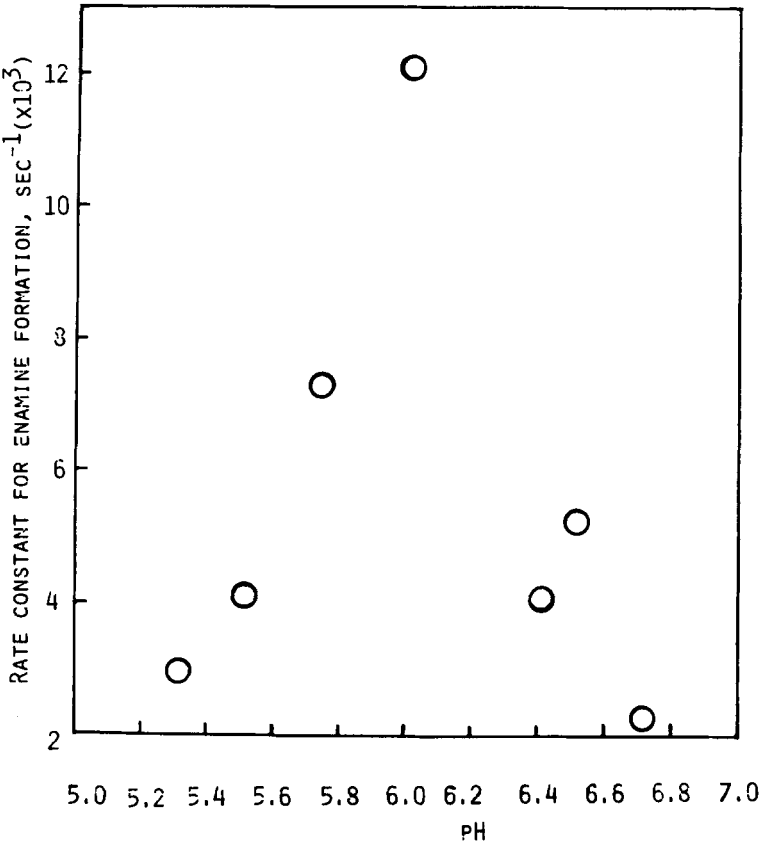

FIGURE 6 pH dependence of the apparent first-order rate constant for build-up of A_{440} produced from PDC–TDP and *m*-NPB at 10°C. Both reference and sample cuvettes contained 0.7 ml "solvent" (see Figure 5), 0.2 ml of 10 mM *m*-NPB (final concentration *ca.* 2 mM) and 0.1 ml mixture of Na_2HPO_4 and NaH_2PO_4 (1 M each) to provide the desired pH. The reaction was initiated by the addition of 0.2 ml (about 30 units total) of PDC to the sample cuvette only. The rate constants were calculated by Guggenheim's method.

Since an enamine derived from such conjugated α-keto acids is long-lived, both enzyme-bound enamine and covalent enzyme-inhibitor complex are inhibitory vis-à-vis pyruvate turnover.

Precise curve fitting and extension of such time resolution studies, as well as more detailed solvent deuterium isotope effect studies will shed further light on the mechanistic details. It is already clear from these preliminary data that the methodology suggested here is capable of providing hitherto unobservable details about thiamin diphosphate dependent enzymatic mechanisms. The direct observation of the fate of the key enamine intermediate will enable us to determine specific rate constants for individual steps, information not readily available by any other current technique.

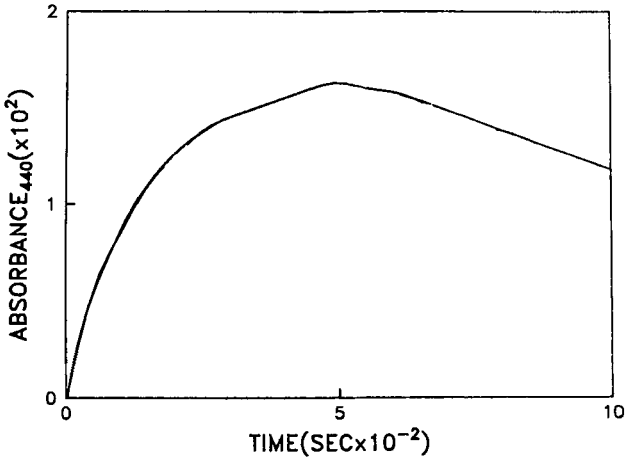

FIGURE 7 Time course of development of A_{440} resulting from mixing PDC · TDP with *m*-NPB at 15°C, pH 5.96. Same experimental conditions as in Figure 6, except only three units of enzyme were used and 0.1 ml of 10 mM *m*-NPB (final concentration *ca.* 1 mM).

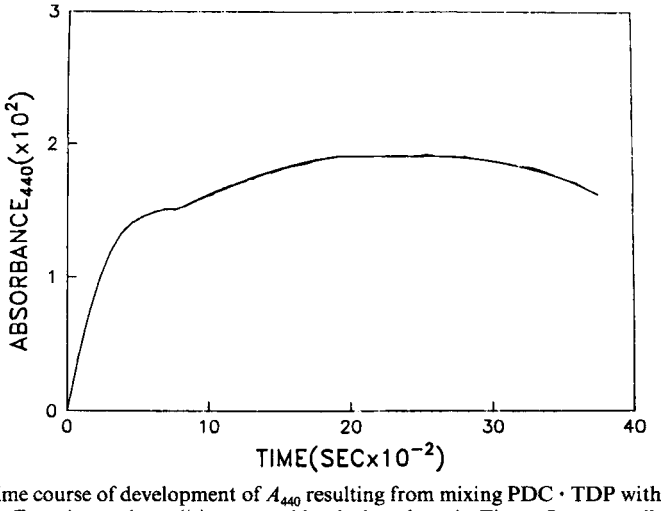

FIGURE 8 Time course of development of A_{440} resulting from mixing PDC · TDP with *m*-NPB at 15°C, pH 6.49 in D_2O. Experimental conditions were identical to those in Figure 7, except all solutions, except the 20 μl enzyme (3 units), were made up in D_2O. pH in D_2O was measured at a glass electrode and is uncorrected.

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