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Active Site Directed Irreversible Inactivation of Brewers' Yeast Pyruvate Decarboxylase by the Conjugated Substrate Analogue (*E*)-4-(4-Chlorophenyl)-2-oxo-3-butenoic Acid: Development of a Suicide Substrate†

Donald J. Kuo and Frank Jordan*

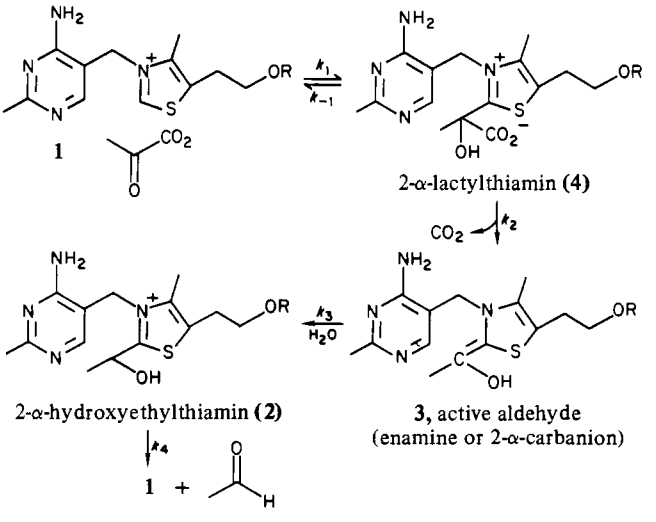
ABSTRACT: (*E*)-4-(4-Chlorophenyl)-2-oxo-3-butenoic acid (CPB) was found to irreversibly inactivate brewers' yeast pyruvate decarboxylase (PDC, EC 4.1.1.1) in a biphasic, sigmoidal manner, as is found for the kinetic behavior of substrate. An expression was derived for two-site irreversible inhibition of allosteric enzymes, and the kinetic behavior of CPB fit the expression for two-site binding. The calculated K_i's of 0.7 mM and 0.3 mM for CPB were assigned to the catalytic site and the regulatory site, respectively. The presence of pyruvic acid at high concentrations protected PDC from inactivation, whereas low concentrations of pyruvic acid accelerated inactivation by CPB. Pyruvamide, a known allosteric activator of PDC, was found to enhance inactivation by CPB. The results can be explained if pyruvamide binds only to a regulatory site, but CPB and pyruvic acid compete for both the regulatory and the catalytic centers. [1-^{14}C]CPB was found to lose $^{14}CO_2$ concurrently with the inactivation of the enzyme. Therefore, CPB was being turned over by PDC, in addition to inactivating it. CPB can be labeled a suicide-type inactivator for PDC.

Thiamin diphosphate (TDP, **1**) was first demonstrated to be an essential cofactor for the nonoxidative decarboxylation of pyruvate by pyruvate decarboxylase (PDC, EC 4.1.1.1; Lohmann & Schuster, 1937). The role of the coenzyme in a variety of other reactions including oxidative decarboxylation of α-keto acids was summarized (Krampitz, 1969; Sable & Gubler, 1982). PDC catalyzes the irreversible decarboxylation of pyruvate, employing tightly bound Mg(II) and TDP as cofactors (Schellenberger, 1967):

$$CH_3COCO_2^- + H_2O \xrightarrow[Mg(II),\ TDP]{PDC} CH_3CHO + CO_2 + OH^- \quad (1)$$

Breslow's model studies demonstrated the importance of the C2 atom of the thiazolium ring in TDP-requiring reactions (Breslow, 1957, 1958). Those model studies were confirmed with the isolation of 2-(1-hydroxyethyl)thiamin diphosphate (HETDP, **2**) from the enzymatic reaction mixtures (Carlson & Brown, 1960; Holzer & Beaucamp, 1961; Krampitz et al., 1961). Scheme I presents a mechanism consistent with the

Scheme I



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0006-2960/83/0422-3735$01.50/0

above information (Breslow, 1958; Jencks, 1969).

On the basis of reports that thiamin thiothiazolone diphosphate is a transition-state analogue for the pyruvate dehydrogenase multienzyme complex (Gutowski & Lienhard, 1976), phenylglyoxalic acid ($C_6H_5COCO_2H$) is a poor substrate for PDC (Schellenberger, 1982), and glyoxalic acid ($CHOCO_2H$) is an irreversible inhibitor of PDC that releases CO_2 into the medium (Holzer & Beaucamp, 1961; Uhlemann & Schellenberger, 1976), we synthesized (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid (CPB). It was assumed that employing CPB with PDC may lead to the formation of a highly conjugated and stable enamine. This enamine may have a potential of being observed spectroscopically (by virtue of the anticipated long-wavelength absorption) and of irreversibly inactivating the enzyme. We here report results confirming that CPB is indeed an irreversible inhibitor of brewers' yeast PDC. Convincing evidence was obtained for two types of binding sites for CPB on PDC, both sites competitive with respect to the substrate pyruvic acid. The data analysis for obtaining irreversible inhibition kinetic constants (Kitz & Wilson, 1962) was extended to two-site allosteric inhibition patterns. The clean behavior of the inhibition patterns also indirectly demonstrated that there must be two types of pyruvate binding sites on PDC. It was also found that in addition to acting as an irreversible inactivator of PDC, CPB was also turned over by the enzyme.

Experimental Procedures

Materials. All chemicals employed were the purest grade commercially available. The potassium salt of CPB was synthesized from 4-chlorobenzaldehyde and pyruvate (Erlenmeyer, 1903; Datta & Daniels, 1963). Elemental analysis (C, H) and proton nuclear magnetic resonance (^1H NMR) (D_2O) were totally consistent with the structure. The *E* configuration with respect to the C=C double bond was deduced from the magnitude of $J_{vicinal}$ = 16 Hz. Aged alumina C^γ was purchased from Sigma Chemical Co., St. Louis, MO.

Synthesis of [1-^{14}C]CPB. A total of 0.5 mg of sodium [1-^{14}C]pyruvate (New England Nuclear, 7.5 Ci/mol) was diluted 15-fold to 8 mg (0.072 mmol) and incubated with 12 mg (0.093 mmol) of 4-chlorobenzaldehyde in 0.5 mL of 2 N NaOH in an ice bath for 1 h. The resulting yellow precipitate was filtered, the solid was dissolved in 3 mL of 0.1 N HCl, and this solution was extracted with 6 × 3 mL of ether. The combined ether layers were dried ($MgSO_4$), and the solvent was evaporated under reduced pressure. Thin-layer chromatography (silica gel) employing 1-butanol/acetic acid/water (4/1/1 v/v) showed that more than 96% of the total counts were in CPB (R_f 0.60) and only trace amounts in pyruvic acid (R_f 0.35).

Demonstration of Turnover of CPB by PDC. The incubation mixtures contained 50 mM citrate, pH 5.0, 0.9 mM [1-^{14}C]CPB (50 mCi/mol from a 1 to 10 dilution of the above), 20 mM pyruvamide, and 34 units/mL PDC. The pyruvamide was employed to enhance the rate of inactivation by CPB (see below); the pH of 5.0 (rather than the optimum of 6–6.2), to minimize nonenzymic decarboxylation of CPB. The incubation was performed at 23 °C. Periodically, 20 μL of the mixture was removed and assayed for activity at pH 6.0 in 3 mL of 0.1 M pyruvic acid at 30 °C. A series of samples were incubated at 23 °C as follows. A total of 240 μL of the above incubation mixture was placed into 1.5-mL centrifuge tubes (Eppendorf). These tubes were then placed upright into 20-mL scintillation counting vials containing 0.5 mL of 1.0 N NaOH and small pieces of filter paper. The vial was then covered with a serum cap and incubated. At the indicated time, 0.5 mL of 50% trichloroacetic acid was injected through the serum cap into the centrifuge tube to quench the reaction, and the sample was set aside for 1.5 h. Next, 0.5 mL of water was used to rinse off the serum camp, and this was added to the NaOH in the vial. Next, 10 mL of Aquasol (New England Nuclear) was added to the vial, and the centrifuge tube was removed carefully from the vial. The remaining solution in the vial (0.5 mL of NaOH, 0.5 mL of H_2O, and 10 mL of Aquasol) was mixed, and the $^{14}CO_2$ released was counted on a Beckman LS-100 scintillation counter. To ensure optimum efficiency and to minimize interference from chemiluminescence, settings from 260 to 420 were employed. In control experiments, PDC was first heat treated (60 °C for 10 min) and then employed at the same conditions as quoted above.

Enzyme Assay. The activity of PDC was determined by the pH-stat method (Leussing & Stanfield, 1966; Schellenberger et al., 1968). Protein content was determined by employing Bradford's (1976) method.

Enzyme Purification. The procedure adopted combined features of protocols reported by Green et al. (1941), Juni & Heym (1968), and Ullrich (1970) and was applicable to the purification of larger amounts of PDC. The specific activities obtained were 3.6 units/mg after the first heat treatment, 20 units/mg after $(NH_4)_2SO_4$ fractionation and the second heat treatment, 33 units/mg after the first alumina C^γ treatment, and 64 units/mg after the second alumina C^γ treatment. According to both sodium dodecyl sulfate tube gel electrophoresis and isoelectric focusing (p*I* ~6.3), a single band constituted about 80% of the total protein.

Results and Discussion

Kinetic Behavior of Conjugated Substrate Analogue CPB. The compound CPB was designed to be an active site directed irreversible inhibitor (Rando, 1974, 1975; Abeles & Maycock, 1976; Walsh, 1977). The rate of inactivation of brewers' yeast PDC by varying concentrations of CPB is presented in Figure 1. A plot of $t_{1/2}$ for inactivation (obtained from the early first-order phase in Figure 1) vs. $[CPB]^{-1}$ or $[CPB]^{-2}$ is presented in Figure 2. The data have been analyzed according to the following scheme:

$$E + CPB \underset{K_{i,r}}{\rightleftharpoons} E\cdot CPB \overset{CPB}{\underset{K_{i,c}}{\rightleftharpoons}} E\cdot CPB_2 \xrightarrow{k_c} E'\cdot CPB_2 \text{ (inactivated enzyme)} \quad (2)$$

where $K_{i,r}$ represents the dissociation constant for CPB from the regulatory site, $K_{i,c}$ is that from the catalytic site, and k_c is the first-order rate constant for irreversible inactivation. An extension of the derivation by Kitz & Wilson (1962) and Jung & Metcalf (1975) for irreversible inactivation with the inclusion of $K_{i,r}$ and with the assumption that [CPB] ≫ E leads to

$$\ln(\epsilon/E_0) = k_c t/(1 + K_{i,c}/[CPB] + K_{i,c}K_{i,r}/[CPB]^2) \quad (3)$$

where E_0 is the stoichiometric amount of enzyme and ϵ is the active enzyme ([E] + [E·CPB] + [E·CPB_2]) remaining at time *t*. The observed half-life for each concentration of inhibitor is

$$t_{1/2} = t_{1/2,\infty}(1 + K_{i,c}/[CPB] + K_{i,c}K_{i,r}/[CPB]^2) \quad (4)$$

(where $t_{1/2,\infty}$ is $0.693/k_c$). At early times, the inactivation followed first-order kinetics and obeyed eq 3. In the latter stages, the kinetics of inactivation became distinctly biphasic. In accord with the predictions from eq 4, a plot of $t_{1/2}$ (obtained from the early stages of inactivation) against $[CPB]^{-1}$

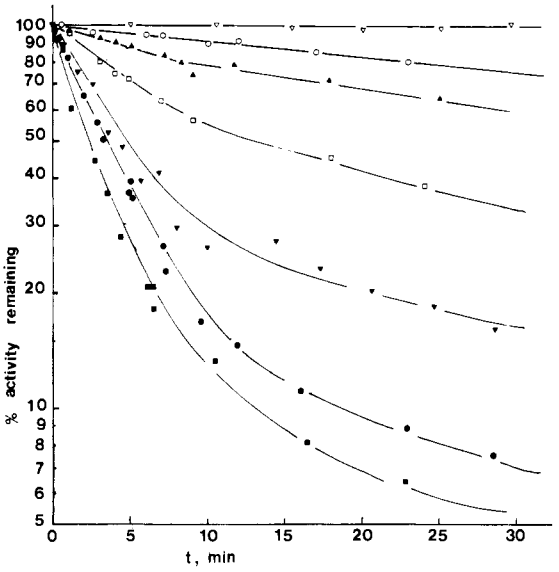


FIGURE 1: Time-dependent inactivation of brewers' yeast pyruvate decarboxylase by CPB. The 0.5-mL samples containing 12 units of PDC, 0.2 mM TDP, 0.2 mM $MgSO_4$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethanesulfonyl fluoride, and 4% (v/v) ethylene glycol in 0.1 M citrate, pH 6.0, were incubated at 30 °C with varying concentrations of CPB [(▽) 0 mM; (○) 0.1 mM; (▲) 0.18 mM; (□) 0.3 mM; (▼) 0.5 mM; (●) 1 mM; (■) 2 mM]. At the indicated time intervals 50-μL aliquots were removed and assayed for PDC activity. Several samples were run in duplicates.

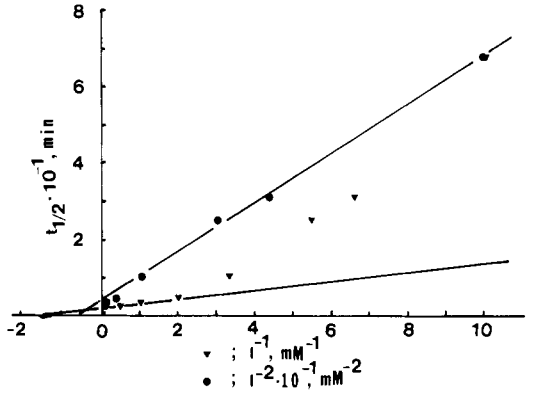


FIGURE 2: Determination of $K_{i,r}$, $K_{i,c}$, and k_c for the CPB-induced irreversible inactivation of PDC. The $t_{1/2}$'s were calculated from the data presented in Figure 1, except for the 0.15 mM CPB data that are not shown in Figure 1. The plots were constructed according to eq 4 in the text by plotting $t_{1/2}$ against $[CPB]^{-1}$ (▼) and $[CPB]^{-2}$ (●), respectively.

was linear for high concentrations of CPB, whereas a plot of $t_{1/2}$ vs. $[CPB]^{-2}$ was linear at low concentration of inactivator. Therefore, the substrate analogue followed the same kinetic pattern as does pyruvic acid (Ullrich & Donner, 1970; Hübner et al., 1970; Uhlemann & Schellenberger, 1976; Schellenberger, 1982). Those authors also reported that PDC is tetrameric, contains four TDP per holoenzyme, and exhibits sigmoidal kinetics with respect to pyruvic acid. The kinetic behavior of CPB therefore confirms that it behaves as does pyruvic acid. The fact that, even upon overnight dialysis against TDP and Mg(II), no activity was regained proved that the inhibition was irreversible. Values of 0.3 mM for $K_{i,r}$, 0.7 mM for $K_{i,c}$, and 0.38 min^{-1} for k_c were obtained (30 °C) for CPB compared to the 0.8 mM and 2 mM K_m's reported for pyruvic acid (Hübner et al., 1978). The lower $K_{i,r}$ and $K_{i,c}$ compared to K_m's are consistent with a hydrophobic binding environment on the enzyme [see Crosby et al. (1970), Crosby & Lienhard (1970), and Jordan et al. (1978) for models and

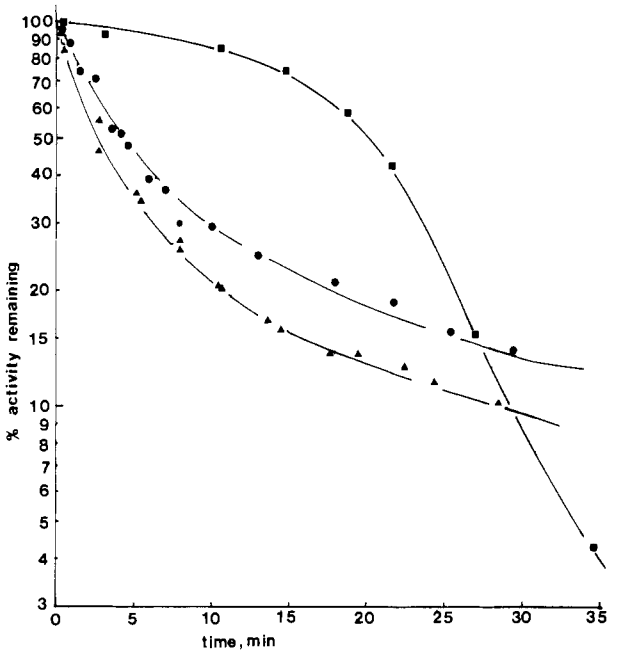


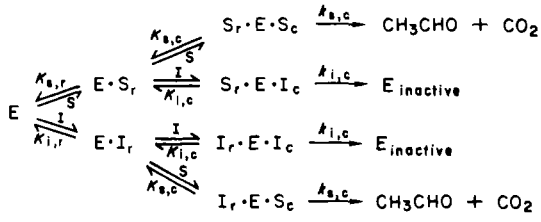
FIGURE 3: Effects of pyruvic acid and pyruvamide on rate of inactivation of PDC by CPB. The 0.5-mL samples contained 12 units of PDC, 0.2 mM TDP, 0.2 mM $MgSO_4$, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.5 mM CPB, and 4% (v/v) ethylene glycol in 0.1 M citrate, pH 6.0 (●), and in the presence of 50 mM pyruvic acid (■) or 30 mM pyruvamide (▲). At the indicated time intervals, 50-μL aliquots were removed and assayed for PDC activity.

Ullrich et al. (1970) and Wittorf & Gubler (1970) for PDC].

Inactivation of PDC by CPB in the Presence of Pyruvic Acid or Pyruvamide. As a further test that CPB is catalytic site directed, the inactivation was also performed in the presence of pyruvic acid. Hübner et al. (1978) reported that pyruvamide or pyruvic acid enhanced the inhibition of PDC induced by glyoxylate. Figure 3 illustrates the inactivation (30 °C) of PDC by CPB alone (●), in the presence of 50 mM pyruvic acid (■), and 30 mM pyruvamide (▲). Pyruvamide is not a substrate and enhances the rate of inactivation by CPB throughout the course of the inactivation. Pyruvic acid, on the other hand, initially protected PDC from inactivation, as expected from the substrate if CPB acted at the catalytic center. At later times, once pyruvic acid was nearly totally consumed, the rate of inactivation increased. These observations are consistent with the existence of two sites for the α-keto acids, one catalytic and one regulatory. Pyruvamide, by contrast, would only bind at the regulatory site. According to this hypothesis, pyruvamide competes successfully with CPB for the regulatory site but not for the catalytic site and makes the latter site more susceptible to inactivation. If ones takes the hypothesis a step further, pyruvic acid appears to compete with CPB successfully for both sites. Once the concentration of pyruvic acid decreases significantly, it resides primarily at the regulatory site and exposes the catalytic site toward inactivation. Just as pyruvate was reported to have a higher affinity for the regulatory site (0.8 mM) than for the catalytic site [2 mM; see Hübner et al. (197)], CPB has a higher affinity for the regulatory (0.3 mM) than for the catalytic center (0.7 mM).

The complex kinetic behavior observed in Figure 3 (■) can be quantified by an extension of eq 2 that includes inactivation of PDC (E) by CPB (I) in the absence and in the presence of pyruvic acid (S) as given in Scheme II. The double indices i and s stand for CPB and pyruvic acid and r and c for regulatory and catalytic sites, respectively. The first-order ap-

Scheme II



parent rate constant for inactivation at any time t, in the presence of pyruvic acid, $k'_{i,c}$ is

$$\ln (\epsilon'/E_0) = -k'_{i,c}t \quad (5)$$

where ϵ' is the concentration of active enzyme remaining at time t in the presence of pyruvic acid ($\epsilon' = [E] + [E{\cdot}I_r] + [E{\cdot}S_r] + [I_r{\cdot}E{\cdot}I_c] + [S_r{\cdot}E{\cdot}S_c] + [I_r{\cdot}E{\cdot}S_c] + [S_r{\cdot}E{\cdot}I_c]$). To simplify the derivation, $k_{s,c}$ was not included. This implies that the derivation applies only to the instantaneous concentration of pyruvic acid at any time t. Also, the condition [I] and [S] $\gg$ [E] is still applicable. An expression for $k'_{i,c}$ in terms of $k_{i,c}$ is given by eq 6.

$$k'_{i,c} = \frac{k_{i,c}}{\frac{K_{i,r}K_{i,c}}{[I]^2} + \left(1 + \frac{K_{i,r}}{[I]}\frac{[S]}{K_{s,r}}\right)\left(1 + \frac{K_{i,c}}{[I]}\left(1 + \frac{[S]}{K_{s,c}}\right)\right)} + \frac{k_{i,c}}{\frac{K_{i,c}}{[I]}\frac{K_{s,r}}{[S]} + \left(1 + \frac{K_{s,r}}{[S]}\frac{[I]}{K_{i,r}}\right)\left(1 + \frac{K_{i,c}}{[I]}\left(1 + \frac{[S]}{K_{s,c}}\right)\right)} \quad (6)$$

By employing $K_{i,c}$ = 0.7 mM, $K_{i,r}$ = 0.3 mM, $K_{s,c}$ = 2 mM, and $K_{s,r}$ = 0.8 mM in eq 6, the ratio, R, of the inactivation rate in the presence compared to the rate in the absence of S [$R = (k'_{i,c}$ at [S])/($k'_{i,c}$ at [S] = 0)] is

[pyruvic acid] (mM)	0.1	0.2	0.3	0.4	0.5
R ([I] = 0.5 mM)	1.0	0.99	0.98	0.98	0.97
R ([I] = 0.1 mM)	1.23	1.40	1.53	1.63	1.70
[pyruvic acid] (mM)	1	2	5	10	50
R ([I] = 0.5 mM)	0.91	0.81	0.54	0.34	0.08
R ([I] = 0.1 mM)	1.82	1.66	1.09	0.67	0.16

Figure 4 presents the rate ratio for inactivation in the presence and absence of S at different times. The dots represent the ratio of the slope in the presence of pyruvic acid [calculated from a tangent to the curve at each experimental value in Figure 3 (■) divided by the initial slope in the absence of pyruvic acid (●)]. The arrows point to the rate ratios listed above that were calculated from eq 6 for different concentrations of pyruvic acid. High concentrations of pyruvic acid offered protection from inactivation. As the concentration of substrate decreased, the rate of inactivation increased until the slope with pyruvate present equaled the initial slope in its absence. Eventually, the rate of inactivation decreased again, as was observed in all other plots in the absence of substrate and in the presence of pyruvamide, due to the onset of the slower biphasic character. Thus the presence of substrate also delayed the onset of the biphasic character in the CPB-induced inactivation. The onset of the biphasic behavior even in the presence of substrate appeared real after about 20 min into the inactivation [there was a change in curvature detectable in Figure 3 (■)]. Implicit in Scheme II is an explanation of the observation that pyruvate enhanced the rate of inactivation of PDC by glyoxylate (Hübner et al., 1978). Under some

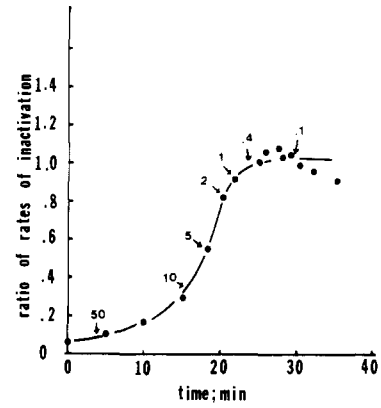


FIGURE 4: Ratio of the rate of inactivation of PDC by CPB in the presence compared to the rate in the absence of pyruvic acid at different times. The data (dots) were obtained by dividing the slope of the tangent to the curve in Figure 3 with pyruvate present [(■) at the indicated times] by the initial slope calculated in the absence of pyruvate (●). The arrows (with concentration of pyruvic acid in mM above) point to the inactivation rate ratios calculated from eq 6 in the text for varying concentrations of pyruvic acid; $K_{i,c}$, $K_{i,r}$, $K_{s,c}$, and $K_{s,r}$ are as listed under Results and Discussion and 0.5 mM CPB.

conditions (see calculated R values for [I] = 0.1 mM), the rate of inactivation in the presence of substrate could exceed the rate in its absence.

The biphasic behavior observed at later times (see Figures 1 and 3) during inactivation of PDC by CPB is well precedented in the literature on suicide-type inactivators including the inhibition of pyridoxal 5′-phosphate dependent enzymes by *N*-hydroxy amino acids (Cooper & Griffith, 1979), the inhibition of L-Dopa decarboxylase by α-(fluoromethyl)-L-Dopa [α-(fluoromethyl)-L-3,4-dihydroxyphenylalanine] (Maycock et al., 1980), and the inhibition of glutamate decarboxylase by α-(fluoromethyl)glutamic acid (Kuo & Rando, 1981). It has been demonstrated by Schellenberger's group (Hübner et al., 1978) that PDC is subject to high substrate activation, and there is a time lag in acetaldehyde release upon addition to pyruvate to PDC. This suggests that PDC may be a hysteretic enzyme (Neet & Ainslie, 1980) with respect to pyruvate. If such a substrate-induced slow conformational change were to take place on PDC, it may explain the onset of the biphasic kinetic behavior apparent in Figures 1 and 3. This hysteretic effect induced by CPB could only be the cause of the observed kinetic behavior if the time scale for the slow conformational change were similar to the time scale for inactivation. Of course another, more trivial, explanation of the biphasic inactivation kinetics is that there are two classes of targets for inactivation with different intrinsic reactivities. These results strongly implicate CPB as a catalytic site directed inactivator and lend further credence to the presence of both regulatory and catalytic sites for pyruvic acid on PDC.

CPB Is both a Substrate for and an Inactivator of PDC. It was important to demonstrate whether CPB was decarboxylated by PDC. When [1-^{14}C]CPB (the specific radioactivity for all experiments was 44.5 cpm/nmol for the counting efficiency employed) was incubated with PDC at pH 5.0, 23 °C, $^{14}CO_2$ was released concurrently with the inactivation of the enzyme (Figure 5). The rapid initial rate of release of CO_2 took on a biphasic character at around 10 min into the inactivation and reached a steady-state rate. This latter rate presumably represented residual enzyme activity due to incomplete inactivation. Since the inactivation rates follow biphasic behavior (Figures 1 and 3), it is not surprising that so does the release of $^{14}CO_2$. An extrapolation of the slower steady-state rate to zero time provides the total number

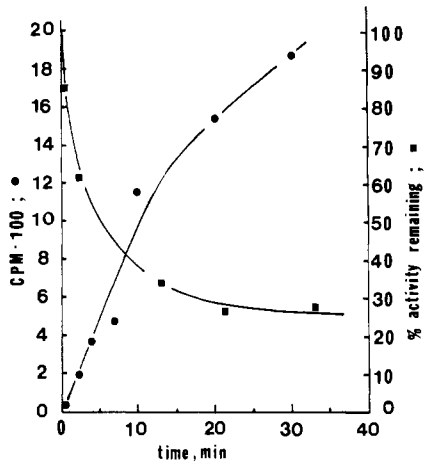
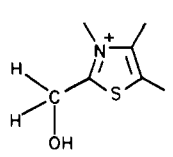


FIGURE 5: Concurrent decarboxylation of and inactivation by [1-^{14}C]CPB. Conditions were those described under Experimental Procedures. The $^{14}CO_2$ cpm quoted represent those counts that resulted by subtraction of the background level that was produced under identical conditions except that the PDC was first heat denatured.

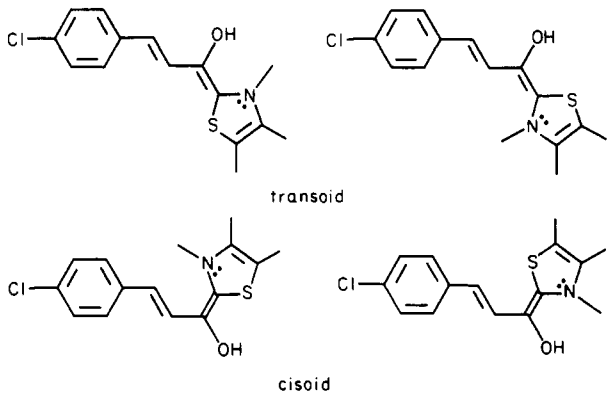
of counts resulting from (i) decarboxylation leading to inactivation and (ii) normal turnover. The 780 cpm (equivalent to 18 nmol of CPB) corresponds to 70% inactivated PDC (1 nmol of catalytically active PDC-bound TDP). If one assumes that 1 mol of CPB is required to inactivate 1 mol of enzyme-bound TDP, the ratio of turnover rate to inactivation rate is 18, i.e., of each 18 molecules of CPB decarboxylated only one inactivates PDC. Similar results were obtained at pH 6.0 (over 95% inactivation observed) with a turnover rate/decarboxylation rate ratio of ca. 70. Control experiments also demonstrated that neither 5 mM 4-chlorocinnamaldehyde (produced by overnight incubation of 5 mM CPB with PDC under conditions that allowed greater than 95% conversion to product according to the $^{14}CO_2$ released) nor 5 mM cinnamaldehyde had any inhibitory effect on PDC. Therefore, the irreversible inactivation by CPB was not due to the ultimate product of decarboxylation. To further demonstrate that CPB is not a group-specific affinity label (as may be anticipated from the presence of the potential Michael acceptor α,β-unsaturated ketone), it was also tested on a totally unrelated enzyme. Even 2 mM concentration of CPB did not inactivate glyoxalase I (an enzyme that also generates a carbanionic intermediate). Therefore, turnover of and inactivation by CPB of PDC must be due to the α-keto acid structure of CPB.

Finally, one can speculate on the mode of inactivation by PDC by CPB. After overnight dialysis, no activity remained with 5 mM initial concentration of CPB present (pH 6.0) but about 31% activity was apparent with 10 mM initial concentration of glyoxylate present (compared to approximately 1% residual activity after 30-min treatment with 5 mM CPB and 19% after 30-min treatment with 10 mM glyoxylate). Therefore, the mode of inhibition by the two compounds is probably different. In analogy with Scheme I, glyoxylate may form a 2-(hydroxymethyl)-TDP, analogous to **2** (Uhlemann



2-(hydroxymethyl)-TDP

& Schellenberger, 1976) that in our hands slowly regenerated active enzyme. CPB, on the other hand, may cause inactivation by first forming one or more of the following four highly resonance-stabilized enamines analogous to **3**:



transoid

cisoid

At least one of the four enamines can be converted to product and released to regenerate active enzyme. At least one other, however, may be hindered from being protonated by an enzymic general acid, hence is not released and perhaps subsequently forms a covalent bond with an enzymic nucleophile. The sum of the evidence suggests that CPB is a mechanism-based suicide-type inactivator (Abeles & Maycock, 1976; Walsh, 1982) and is the first reported one for any thiamin diphosphate requiring enzymatic process.

Acknowledgments

We are grateful to the Anheuser-Busch Brewing Co., Newark, NJ, for their continued generosity in supplying the brewers' yeast employed in our studies.

Registry No. PDC, 9001-04-1; CPB, 42393-06-6; [1-^{14}C]CPB, 86088-42-8; pyruvamide, 631-66-3; sodium [1-^{14}C]pyruvate, 7540-66-1; 4-chlorobenzaldehyde, 104-88-1.

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Stoichiometry of Phenylhydrazine Inactivation of Pig Plasma Amine Oxidase†

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ABSTRACT: Pig plasma amine oxidase is irreversibly inactivated by phenylhydrazine. The stoichiometry of this inactivation was determined by monitoring the loss of catalytic activity, the formation of a new visible spectral band, changes in the circular dichroic spectrum and by equilibrium binding studies. In all cases, only 1 mol of phenylhydrazine reacted with the dimeric pig plasma amine oxidase; further additions of phenylhydrazine had no effect. Pretreatment of the enzyme with phenylhydrazine inhibited the binding of amine substrate. The phenylhydrazine–enzyme complex was found to be stable under various experimental conditions for at least 72 h. Circular dichroic spectra revealed the conformation of the phenylhydrazine-treated enzyme to be altered in the region around prosthetic groups and indicated some changes about the aromatic amino acids. No major conformational changes were detected by this technique. Isoelectric focusing experiments exposed no differences in the band pattern or isoelectric point between the untreated and phenylhydrazine-treated enzymes.

Plasma amine oxidase (PAO)[1] from pig[2] is one of a family of soluble amine oxidases containing Cu^{2+} ions and an organic prosthetic group capable of interacting strongly with carbonyl reagents (Buffoni & Blaschko, 1964; Blaschko & Buffoni, 1965; Buffoni, 1968). The molecular weight of pig PAO has been reported between 186 000 and 196 000 (Buffoni & Blaschko, 1964; Boden et al., 1973; Barker et al., 1979). In the presence of denaturing reagents, it behaves as a single species of molecular weight 95 000–97 000 (Boden et al., 1973; Barker et al., 1979). Barker et al. (1979) suggest the subunits have identical primary structure on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing under denaturing conditions. The closely related bovine PAO has been shown to be composed of two identical subunits by tryptic peptide mapping (Achee et al., 1968).

There is suggestive evidence that the "active-carbonyl" cofactor is pyridoxal phosphate or a modified form of pyridoxal phosphate (Malmstrom et al., 1975). Reaction of the active-carbonyl cofactor with hydrazine and other carbonyl reagents inactivates the enzyme competitively with amine substrates (Lindstrom et al., 1974). Lindstrom & Pettersson (1973, 1978) have demonstrated that hydrazine reagents irreversibly inactivate pig PAO with a stoichiometric ratio of 1 mol of hydrazine per 196 000 g of PAO. Anaerobic titration of pig PAO with substrates yields the same ratio (Lindstrom & Pettersson, 1978; Massey & Churchich, 1977). This contradicts the ratio of 3 mol of phenylhydrazine per 195 000 g of PAO of Buffoni & Ignesti (1975), the 3 mol of [^{14}C]histamine bound per mol of enzyme in the form of a Schiff base (Buffoni, 1968), and the 3.8 mol of phosphate bound per dimer

† From the Naval Medical Research Institute, Bethesda, Maryland 20814. *Received July 28, 1982; revised manuscript received January 7, 1983.* This research was supported by the Naval Medical Research and Development Command (Research Task No. MR0412001.0436). The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or as reflecting the views of the Navy Department or the naval service at large.

[1] Abbreviations: PAO, plasma amine oxidase (from pig); Temed, *N,N,N'N'*-tetramethylethylenediamine; Bis, *N,N'*-methylenebis(acrylamide); CD, circular dichroism; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance.

[2] Amine:oxygen oxidoreductase (deaminating) (EC 1.4.3.6).