Reactivity at the Substrate Activation Site of Yeast Pyruvate Decarboxylase: Inhibition by Distortion of Domain Interactions†

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ABSTRACT: The residue C221 on pyruvate decarboxylase (EC. 4.1.1.1) from *Saccharomyces cerevisiae* has been shown to be the site where the substrate activation cascade is triggered [Baburina et al. (1994) *Biochemistry 33*, 5630-5635] and is located on the β domain [Arjunan et al. (1996) *J. Mol. Biol. 256*, 590], while the active-center thiamin diphosphate is located >20 Å away, at the interface of the α and γ domains. The reactivity of all three exposed cysteines (152, 221, and 222) was examined under the influence of known activators and inhibitors. Protein chemical methods, in conjunction with [1-^{14}C] and [3-^{3}H] analogues of the mechanism-based inhibitor *p*-$ClC_6H_4CH{=}CHCOCOOH$, demonstrated that the holoenzyme bound approximately 2−3 atoms of tritium/atom of C-14. However, when the labeled enzyme was subjected to trypsinization, followed by sequencing of the labeled peptide, only the tritium label was in evidence at C221, with a stoichiometry of 2 atoms of tritium/tetrameric holoenzyme. Apparently, the product of decarboxylation bonded to the enzyme survived the limited proteolysis and sequencing, but the bound 2-oxoacid was released during the protocol. Surprisingly, the C221S or C222A variants, although they still possess 20−30% specific activity compared to the wild-type enzyme, could still be inhibited by the $XC_6H_4CH{=}CHCOCOOH$ class of inhibitors/substrate analogues, as well as by the product of decarboxylation from such compounds, cinnamaldehydes. Other potential nucleophilic sites for the inhibitor [C152 (the third exposed cysteine), residues D28, H114, H115, and E477 at the active center and H92 at the regulatory site] were also substituted by a nonnucleophilic side chain. All variants were still subject to inhibition by *p*-$ClC_6H_4CH{=}CHCOCOOH$, the active-center variants being inactivated even faster than the wild-type enzyme, suggesting that the active center is involved in the inactivation process. It appears that C221 is one of only two sites of interaction with such compounds (perhaps the result of a Michael addition across the C=C bond), yet the bound [1-^{14}C]-labeled inhibitor could no longer be detected after peptide mapping at this site or at the catalytic site. Upon combining the tritiated inhibitor with [2-^{14}C]-thiamin diphosphate, no evidence could be found for a thiamin−inhibitor−protein ternary complex, suggesting that the thiamin-bound enamine intermediate did not react further with the protein. It is likely that the second form of inhibition is at the active center, with the inhibitor cofactor-bound, which would have been released during the proteolytic protocol. Among other known activators, ketomalonate was found to react at C221 only. Glyoxalic acid, a mechanism-based inhibitor, on the other hand, could react at both the regulatory and the catalytic center. The high reactivity of C221 is consistent with it being in the thiolate form at the optimal pH of the enzyme [forming a Cys221S$^-$ $^+$HHis92 ion pair; see Baburina et al. (1996) *Biochemistry 35*, 10249−10255, and Baburina et al. (1998) *Biochemistry 37*, 1235−1244]. Several additional compounds were tested as potential regulatory site-directed reagents: iodoacetate, 1,3-dibromoacetone, and 1-bromo-2-butanone. All three compounds reduced the Hill coefficient and hence appear to react at C221. It was concluded that either substitution of C221 by a nonnucleophilic residue *or* large groups attached to C221 in the wild-type enzyme lead to a distortion of domain interactions, interactions which are required for both optimal activity and substrate activation.

Pyruvate decarboxylase from yeast (PDC,[1] EC 4.1.1.1) is one of several enzymes that participate in nonoxidative functions of thiamin diphosphate (ThDP, the vitamin B1 coenzyme). PDC converts pyruvate to acetaldehyde and carbon dioxide [for reviews, see Krampitz (1969), Sable and Gubler (1982), Kluger (1987), Schellenberger and Schowen (1988), Bisswanger and Ullrich (1991), and Bisswanger and Schellenberger (1996)]. The enzyme also has a requirement

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[1] Abbreviations: ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase (EC. 4.1.1.1); scpdc1, wild-type pyruvate decarboxylase isolated from *Saccharomyces cerevisiae*; supdc1, wild-type pyruvate decarboxylase isolated from *Saccharomyces uvarum*; WT, wild-type pyruvate decarboxylase; C221S, C222S, C221S/C222S, C152A, and E477A, are variants of this enzyme; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; CPB, (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid; n_H, Hill coefficient; TPCK-treated trypsin, tosylphenylalanylchloromethyl ketone-treated trypsin; NPB, (*E*)-4-(4-nitrophenyl)-2-oxo-3-butenoic acid; NA, 4-nitrocinnamaldehyde; TB, (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SLG, *S*-lactoylglutathione; pCMB, p-chloromercuribenzoic acid.

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for a divalent cation best fulfilled by Mg(II). PDC from yeast is subject to substrate activation (Boiteux & Hess, 1970; Hübner et al., 1978). In a series of earlier contributions [see Baburina et al. (1998) for background], it was demonstrated that Cys221 at >20 Å from ThDP serves as the site at which substrate activation is triggered (Zeng et al., 1993; Baburina et al., 1994, 1996, 1998). In the accompanying paper results were presented showing that information from C221 in the β domain is transmitted to the active center across the domain divide to H92 on the α domain and that conversion of H92 to uncharged residues results in much reduced positive cooperativity and reduced long-term stability. The experiments reported herein were carried out to assign the mechanism and site of interaction of several different inhibitors/activators/alternate substrates with PDC, not clarified to date. Extensive evidence is presented that C221 and the active-center ThDP are the most, and essentially exclusive, reactive sites on the enzyme and that several reagents previously used in kinetic studies, in fact, interact with both sites. The results underline the importance of unperturbed domain−domain interactions to both full activity and regulation of this enzyme.

EXPERIMENTAL PROCEDURES

The construction of vectors and overexpression of C221S, C222S singly and C221S/C222S doubly substituted variants (Baburina et al., 1994), and of the C152A and H92A variants have been reported elsewhere (Baburina et al., 1996, 1998). Restriction enzymes were purchased from Promega and New England Biolabs. Sequencing was performed on an Applied Biosystems Model 373 DNA sequencer using a dye-terminator sequencing kit.

Materials. *p*-Nitrocinnamaldehyde (NA) was purchased from Aldrich. Glyoxalic acid, ketomalonate, DTT, and *p*CMB were purchased from Sigma. Iodoacetate was purchased from Acros. DTNB was purchased from Kodak. ^3H_2O (1.0 Ci/g) and [1-^{14}C]pyruvate (sodium salt, 11.9 mCi/mmol) were obtained from New England Nuclear. [2-^{14}C]-Thiamin hydrochloride (16.9 mCi/mmol) was a gift of Richard Muccino of Hoffmann-La Roche Inc. of Nutley, NJ. Brewer's yeast (*Saccharomyces uvarum*) slurry was generously supplied by Anheuser-Busch, Newark, NJ.

Purification of PDC. PDC from *S. uvarum* (supdc1, the strain received from Anheuser-Busch) was purified into its α_4, $\alpha_2\beta_2$, and β_4 isoforms, while PDC from *Saccharomyces cerevisiae* (scpdc1) was purified into its α_4 complex by previously described procedures (Kuo et al., 1986; Farrenkopf & Jordan, 1992). All experiments utilized the α_4 isoform of supdc1 and scpdc1, except where noted. PDC was assayed by the pH-stat (Schellenberger et al., 1968) or the NADH/alcohol dehydrogenase coupled assay (Holzer et al., 1956).

Synthesis of [1-^{14}C]CPB, [3-^{3}H]CPB, and [2-^{14}C]thiamin diphosphate is reported in the supporting information.

Labeling of PDC with [3-^{3}H]CPB. A solution of [3-^{3}H]-CPB was prepared by dissolving 2−3 mg of [3-^{3}H]CPB in an appropriate amount of 10 mM citrate, pH 6.0, to give a final concentration of 20 mM [3-^{3}H]CPB. The α_4 isoform of supdc1 was used in all labeling experiments. Enzyme activity was determined with a Radiometer pH-stat system (pH end point = 6.0), except where noted. A reaction mixture containing the following components was prepared: 0.1 M sodium citrate, pH 6.0, 0.2 mM ThDP, 0.2 mM $MgCl_2$, 0.1 mM EDTA, 20% ethylene glycol, 120 units of PDC, and 0.1 mM PMSF; and to initiate inactivation and labeling of the enzyme, 2 mM of tritiated inhibitor was added. The reaction was allowed to proceed until 90−97% inhibition resulted. Then the reaction mixture was placed into two Amicon Centricon 10 (or 30) microconcentrators, diluted to 2.0 mL with 10 mM sodium citrate, pH 6.0, and subjected to five desalting centrifugations/dilutions to remove most of the unbound tritiated substrate and/or products. After the final centrifugation, the sample was recovered (1.0−1.5 mL) and then applied to either a 10 or 30 mL Sephadex G-25M column and eluted with 10 mM citrate, pH 6.0, to further remove any ^{3}H not associated with the enzyme. Fractions of 1 mL were collected and A_{280}, radioactivity, and protein content (Bradford, 1976) of selected fractions were determined. The stoichiometry of inhibitor to enzyme was determined by the ratio of radioactivity in disintegrations per minute per unit of protein. Fractions containing >100 μg/mL protein were lyophilized and stored at −80 °C.

Identification of the Amino Acid Residue Modified by CPB. A sample combining lyophilized G-25 fractions of tritiated PDC samples (2.49 mg of protein) was dissolved in 415 μL of 100 mM NH_4HCO_3/CH_3CN (95:5) (Tempst et al., 1990). TPCK-treated trypsin (20:1 PDC:trypsin) was added and the solution was placed in a water bath at 37 °C for 6 h and then frozen at −20 °C. On the next day the sample was reduced with 2-mercaptoethanol and then alkylated with 4-vinylpyridine (0.6%) for 30 min at room temperature in the dark (Tempst et al., 1990). Immediately following treatment of the sample with 4-vinylpyridine, the sample was diluted with 0.7 mL of 0.1% trifluoroacetic acid (TFA) and injected onto a Vydac C-18 semipreparative column (10 mm × 25 cm). All subsequent HPLC analyses conducted with the Vydac C-18 semipreparative column utilized the chromatographic conditions described in Figure 1A except where noted. One minute fractions were collected and aliquots were removed from each fraction for measurement of radioactivity. Fractions 88−91 contained the maximum amount of radioactivity. These fractions were combined, reduced in volume to approximately 1.0 mL, and injected onto a Vydac C-18 analytical column (4.6 mm × 25 cm; see Figure 1B for flow rate and gradient conditions). The fraction containing the peak amount of radioactivity (99) was sequenced at Bristol-Myers Squibb in Syracuse, NY.

N-Terminal Sequencing. The sequence of amino acids of a 100 μL sample was determined by automated Edman degradation on an Applied Biosystems (ABI) 477A protein sequencing system using the ABI 610 data analysis software. The phenythiohydantoin-derivatized (PTH) amino acids, including tryptophan and pyridylethylcysteine, were resolved on-line by HPLC. PTH-cysteine is not detected. The chromatograms for a series of cycles were compared and an amino acid was determined to occur at a particular position by comparison of the relative amounts of that amino acid among consecutive chromatograms (peak heights and integrated picomole amounts were examined). When more than one peptide was present, the picomole amount of each PTH-amino acid was used to determine the most likely sequence and this was subsequently compared to the protein sequence deduced from the DNA sequence.

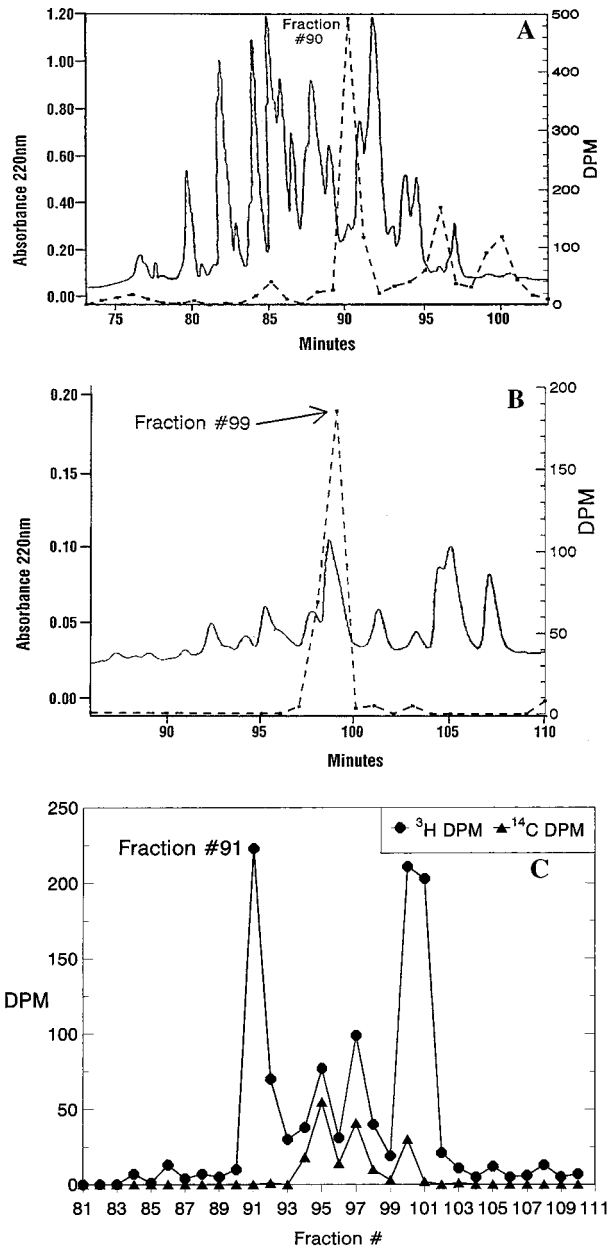


FIGURE 1: (A) Reverse-phase HPLC analysis of tryptic digest of PDC inhibited by [3-^{3}H]CPB. Eluents used were 0.1% TFA (buffer A) and 0.1% TFA in acetonitrile (buffer B). The following gradient conditions were utilized: 0−60 min, 5% buffer B; 60−120 min, 5−100% buffer B. The eluent was monitored at 220 nm, 2.0 absorbance units at full scale (AUFS), and the column was eluted at 1.5 mL/min. Only the section of the chromatogram displaying absorbance peaks corresponding to tryptic peptides is shown. Aliquots (0.3 mL) of each fraction were removed to measure radioactivity, which is represented by the dashed line. (B) Reverse-phase HPLC analysis of concentrated fractions 88−91 (from panel A). With a Vydac C-18 analytical column (4.6 mm x 250 mm) and the same eluents as used in panel A, the sample was eluted using the following chromatographic conditions: 0−120 min, 0−35% buffer B, 0.8 mL/min, absorbance at 220 nm and 2.0 AUFS. Only that portion of the chromatogram displaying absorbance peaks is shown. Aliquots (0.3 mL) of each fraction were removed to determine radioactivity, which is represented by the dashed line. (C) Radioactivity of fractions collected following reverse-phase HPLC analysis of tryptic digest of combined G-25 fractions. Chromatographic conditions are identical to those described in panel A. Aliquots (0.4 mL) were removed from each fraction to determine ^{3}H and ^{14}C contents and only that portion of the chromatogram corresponding to the fractions containing peak radioactivity (fractions 81−111) is shown.

Simultaneous Treatment of PDC with [1-^{14}C]CPB and [3-^{3}H]CPB. This experiment was conducted to determine if the carboxylic acid group of CPB was retained on reaction of PDC with CPB and to determine the stoichiometry of each labeled inhibitor to PDC. Separate solutions containing [1-^{14}C]CPB and [3-^{3}H]CPB were prepared with sufficient 10 mM citrate, pH 6.0, to yield a combined inhibitor concentration of approximately 20 mM. A final inhibitor concentration of 2 mM was used to inhibit PDC. A reaction mixture containing the following components was prepared: 0.1 M sodium citrate, pH 6.0, 0.4 mM ThDP, 1.0 mM $MgCl_2$, 0.1 mM EDTA, 20% ethylene glycol, and 287 units of PDC (scpdc1, 41 units/mg). After addition of these components, 0.479 mL of the dual label inhibitor mix was added to a final volume of 5.0 mL to yield 600 000−700 000 dpm/mL of each labeled inhibitor. The reaction and removal of unbound labeled compounds was conducted as described for the labeling experiments utilizing [3-^{3}H]CPB. The sample was chromatographed on a G-25 column, 1 mL fractions were collected, and the radioactivity in each fraction was determined by adding 0.4 mL aliquots of each fraction to a 20 mL scintillation vial to which 10 mL of Ecolume was added. The ^{3}H and ^{14}C contents (in disintegrations per minute) were determined using a computer-generated dual label standard curve (stored in a Packard 300C liquid scintillation counter), which was established using quenched aliquots of ^{3}H-toluene and ^{14}C-toluene standards. Those fractions containing >100 μg of protein were lyophilized and subjected to trypsin digestion, reduction, and alkylation as previously described. The digest was subjected to peptide analysis utilizing the Vydac C-18 semipreparative column and eluting solvents as described above and 1 min fractions were collected. Aliquots (0.4 mL) of fractions were taken to determine ^{3}H and ^{14}C disintegrations per minute of each fraction. Those fractions containing peak amounts of ^{14}C and absorbance at 220 nm were subjected to peptide sequencing.

Determination of the Type of Bond Formed between [3-^{3}H]-CPB and PDC. Once sequence analysis suggested that [3-^{3}H]CPB had reacted with a cysteine, an experiment was carried out to ascertain the nature of the bond formed between the inhibitor and the cysteine. The fraction containing the labeled inhibitor bound to C221 (fraction 91, Figure 1C) from HPLC of the tryptic digest of PDC reacted with [3-^{3}H]CPB and [1-^{14}C]CPB was reduced to 0.5 mL on a Savant Speed-Vac to remove acetonitrile and TFA. The sample was then diluted to 1.0 mL with H_2O. As a control, 5 mg of *S*-lactoylglutathione (SLG), a thiol ester, was dissolved in 5.0 mL of 0.1% TFA in acetonitrile/H_2O (50:50) to approximate the solvent composition at which the tritiated peptide was eluted. Two tubes containing 1 mL each of the SLG solution along with two tubes containing only 1 mL of the solvent were then reduced in volume to 0.5 mL and similarly diluted to 1 mL with H_2O. The presence of a thiol ester would be indicated by its reaction with NH_2OH (Flournoy & Frey, 1989) and the release of free thiols. Duplicate aliquots of 0.5 mL of each of the above solutions were then reacted with 0.5 mL of NH_2OH (resulting in a final NH_2OH concentration of 1 M) for 1 h at ambient temperature. Following the reaction, the tubes containing the tritiated peptide were frozen at −80 °C. The contents of the other tubes were then assayed for the presence of thiols

using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to determine the effectiveness of the hydroxylamine treatment (Ellman, 1959). The frozen sample containing the tritiated peptide was thawed, mixed with 1.0 mL of 0.1% TFA, and injected onto the Vydac C-18 semipreparative column. The same buffer system was used as in the initial analysis of the tryptic digest, and 1.0 min fractions were collected and aliquots were taken to determine if the [^{3}H]peptide eluted at the same time as in the original analysis.

Reconstitution of ApoPDC with [2-^{14}C]ThDP-PDC and Subsequent Reaction with [3-^{3}H]CPB. PDC ($\alpha_2\beta_2$ isoform, supdc1) was resolved from its cofactors—ThDP and Mg(II)—by mixing 6−7 mg (approximately 1.0 mL) of enzyme with 1.0 mL of dissociation buffer (DB) containing 20 mM Tris, pH 8.5, 1 mM EDTA, and 0.5 mM PMSF. This solution was placed in a Centricon 30 microconcentrator and centrifuged for 25 min at 6000 rpm at 4 °C. The volume of the apoenzyme solution was reduced to 0.4−0.5 mL. The sample was diluted to 2.0 mL with DB and centrifuged and diluted three more times to ensure removal of cofactors. Following the final centrifugation, the sample was then diluted to 2.0 mL with reconstitution buffer (RB) containing 50 mM citrate, pH 6.3, 1 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), and 0.5 mM PMSF. The microconcentrator was centrifuged for 25 min at 6000 rpm at 4 °C. The volume of the solution was reduced to 0.4−0.5 mL. The sample was diluted to 2.0 mL with RB and centrifuged and diluted three more times to ensure lowering of pH to 6.3 and reconstitution with Mg(II). After the final centrifugation, approximately 800 μL of the partially reconstituted PDC was recovered. A 50 μL sample of this solution was assayed and showed no activity, indicating complete removal of ThDP. Sufficient [2-^{14}C]ThDP was then added to the solution (from a stock solution of [2-^{14}C]ThDP at 35−50 mM in 0.01 N HCl) to give a final concentration of [2-^{14}C]ThDP of 0.4 mM. The solution also contained 50 mM citrate, pH 6.3, 1 mM $MgCl_2$, 0.1 mM EDTA, and 20% ethylene glycol in a final volume of approximately 2.70 mL. About 80% of the activity was recovered, with maximum enzyme activity regained after 40−45 min. At that time, sufficient [3-^{3}H]CPB was added to give a solution that was 2 mM in [3-^{3}H]CPB. The reaction and removal of unbound labeled compounds again was conducted as described for the labeling experiments utilizing [3-^{3}H]CPB. Fractions of 1 mL were collected from a G-25 desalting column and the A_{280} and radioactivity per fraction were determined. The ^{3}H and ^{14}C contents of each fraction were determined utilizing the same dual label standard curve described earlier.

Peptide Analysis of the [3-^{3}H]CPB−[2-^{14}C]ThDP−PDC Complex. To determine if a stable ternary complex was formed among CPB, ThDP, and a residue on PDC, the following analysis was performed. First, 100 nmol of ThDP was injected onto the C-18 semipreparative column to determine where ThDP would elute. Next, lyophilized G-25 fractions containing the [3-^{3}H]CPB−[2-^{14}C]ThDP−PDC complex were combined and the sample was subjected to trypsin digestion, reduction, and alkylation as previously outlined. Following alkylation, the digest was immediately injected onto a Vydac C-18 semipreparative column and the peptides were separated as previously described. One minute fractions at 1.5 mL/min were collected and ^{3}H and ^{14}C disintegrations per minute of each fraction were determined.

Synthesis of (E)-4-(4-Nitrophenyl)-2-oxo-3-butenoic Acid. NPB was synthesized according to a method used to synthesize (*E*)-4-(*p*-tolyl)-2-oxo-3-butenoic acid (TB; Annan et al., 1989). KOH (2.52 g) dissolved in ethanol (20 mL) was added dropwise over 20 min to a stirred mixture of 4-nitrobenzaldehyde (2.26 g) and pyruvic acid (0.03 mol dissolved in 30 mL of methanol). Stirring was continued for 6 h. The resulting yellow potassium salt was filtered after cooling on an ice bath and washed with cold methanol. Next, it was dissolved in water and acidified with 2 N H_2SO_4 and then extracted with ether (3 × 75 mL). The combined ether layers were dried (Na_2SO_4) and the ether was removed with the rotary evaporator. Analysis by TLC (aluminum oxide on aluminum plate, F254 [neutral type] using the solvent system butanol/H_2O/HAc 4:1:1) showed a single spot, and proton NMR revealed a purity of >90%; ^{1}H NMR (500 MHz, CD_3CN/TMS) δ 8.263 (d, 2 H, J = 8.5 Hz), 7.940 (d, 2 H, J = 8.5 Hz), 7.933 (d, 1 H, J = 16 Hz), and 7.569 (d, 1 H, J = 16 Hz).

Inhibition Studies. The indicated reagent was dissolved in a total volume of 1 mL containing 0.1 M MES, pH 6.0; 1 mM ThDP; 2 mM Mg^{2+}, and 1 mM EDTA at 25 °C. Aliquots were removed and then assayed for activity, the activity at zero time being considered as 100%. In cases of time-dependent inactivation, a control reaction containing all components (including the solvent used to dissolve the inhibitor, if other than the buffer) except for the inhibitor itself was used to monitor the activity throughout the experiment. In typical experiments, ∼30 units of PDC or its variants was incubated with different concentrations of inhibitor. ThDP concentration in the range of 10−100 mM had no effect on the inactivation.

Enzyme Dilution and Dialysis. Inactivated enzyme was dialyzed against 100 volumes of the same incubation buffer containing no inhibitors or diluted 100 times with the same buffer. The activity of the inhibited enzymes was checked several times within 5 min of completion of the experiment to make certain that the activity remained stable.

Stock Reagents. All inhibitor solutions were prepared fresh on the day of the experiment. CPB was prepared as a 20 mM stock in 0.1 M MES buffer, pH 6.0; NPB was prepared as a 100 mM stock in 0.1 M MES buffer, pH 6.0; NA was prepared as a 50 mM stock in acetonitrile. All controls for NA contained the appropriate amount of acetonitrile in the mixture. Acetonitrile at these concentrations (< 0.5%) did not alter the PDC activity.

Glyoxalic acid was prepared as a 100 mM stock in 0.1 M MES buffer, pH 6.0. Ketomalonate is less soluble in aqueous solutions and was used out of a 20 mM stock. DTNB was used as a 100 mM stock in methanol. All inactivation experiments with this reagent contained the appropriate amount of methanol in the control mixture. Methanol at low concentrations does not influence the activity of PDC. DTT was prepared fresh daily in an aqueous buffer.

Analysis of Inhibition Data. The activity remaining with time was measured for incremented inhibitor concentration. $t_{1/2}$ (or k_{app}) can be estimated from semilogarithmic plots of the remaining activity vs time. A plot of $1/k_{app}$ vs 1/[I] should give a straight line for noninteracting sites. If the line goes through the origin it indicates no formation of a reversible complex with the enzyme prior to irreversible inactivation (Kitz & Wilson, 1962), while intersection on

the positive *y*-axis indicates that the inhibitor can saturate the enzyme prior to inactivation. A plot of % activity vs [I], if linear, suggests single-site modification of the enzyme; curved dependence implies inhibition at multiple sites (Segel, 1975) or depletion of inhibitor, which in some cases is also a substrate.

RESULTS AND DISCUSSION

(A) Interaction of PDC with CPB, NPB, and Cinnamaldehydes

Since 1983, there have appeared several reports from this laboratory confirming that CPB and related compounds are both substrates and time-dependent inactivators of PDC. It was later found that such compounds inhibit PDCs from a variety of organisms. Below are presented experiments first using protein chemical means and then site-directed variants in an attempt to define the loci of interaction between CPB and PDC.

Identification of the Amino Acid Residue of PDC Modified by CPB. With [3-^{3}H]CPB a ratio of 1.48 ± 0.22 mol of tritiated inhibitor/mol of enzyme was determined (four determinations). When corrected for fully active enzyme (assuming 50 units/mg for fully active pure PDC), a stoichiometry of approximately 2 mol of inhibitor/mol of tetrameric PDC is obtained. The results from the initial HPLC analysis of the tryptic digest of PDC reacted with [3-^{3}H]CPB utilizing the semipreparative column are shown in Figure 1A. Fractions 88−91, which encompassed the major tritium fraction (90) and contained 42.4% of the radioactivity eluted from the column, were pooled, concentrated, and applied to the analytical C-18 column. The UV chromatogram and ^{3}H content of the fractions are shown in Figure 1B. Fraction 99 (containing 56.4% of the radioactivity applied to the column) was subjected to peptide sequencing and the following two peptides were clearly identified: peptide 1, N-P-V-I-L-A-D-A-doublet-PEC-S-R, and peptide 2, L-M-D-L-T-Q-F-V-I-L-V-T, in a mass ratio of 3:1 according to sequence analysis. Peptide 1 is identical with residues 213−224 in the amino acid sequence of PDC1 from *S. cerevisiae* (Hohmann & Cederberg, 1990), with two exceptions. The ninth sequencing cycle, which would correspond to position 221, reveals an unidentified doublet. The tenth cycle, corresponding to residue 222, shows the presence of pyridylethylcysteine (PEC). Positions 221 and 222 are occupied by cysteine residues in scpdc1. Peptide 2 could be matched to amino acid sequence 234−243. A third peptide that was in much lower concentration than peptide 1 or 2 could not be matched to any known sequence in scpdc1.

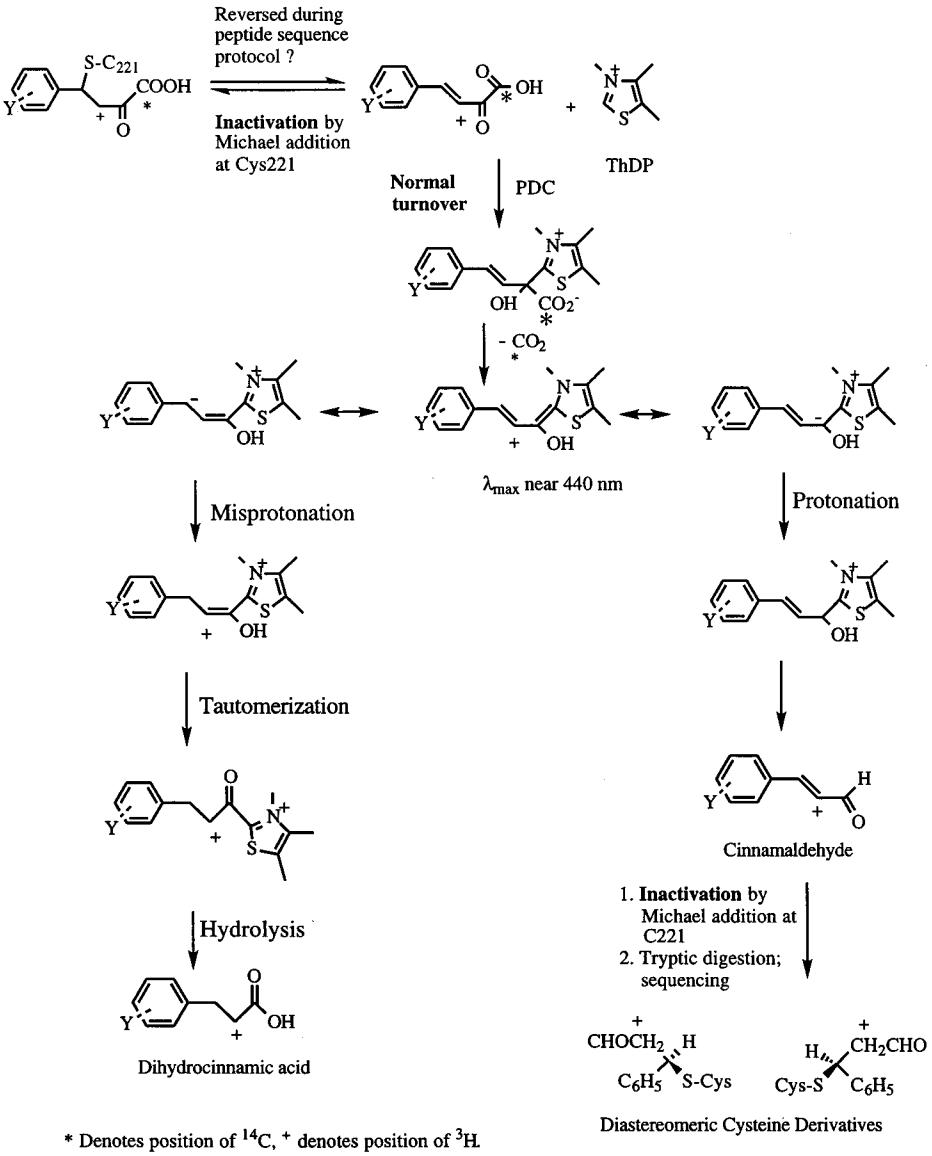
Reaction of [1-^{14}C]CPB and [3-^{3}H]CPB with PDC. When PDC was reacted simultaneously with [1-^{14}C]CPB and [3-^{3}H]-CPB, the mean ratios obtained in the collected fractions were as follows (n = 4 for each): [3-^{3}H]CPB:PDC = 2.35 (SD = 0.14); [1-^{14}C]CPB:PDC = 0.8 (SD = 0.14). Since some residual ^{14}C remained associated with PDC, these fractions were pooled and subjected to trypsin digestion and peptide analysis (on the semipreparative column as previously described), to determine if nondecarboxylated inhibitor had reacted with an amino acid residue. The radioactivity of the fractions from that analysis is shown in Figure 1C. Fraction 91 contained the peak amount of ^{3}H, which earlier work in this study had revealed is associated with a peptide encompassing positions 213−224. Notably, there was no ^{14}C associated with this fraction. The other major radioactive peak was found in fractions 100 and 101. The presence of such a large amount of label in these particular fractions is attributed to undigested PDC, which elutes at this position (data not shown). Fractions 95 and 97 did contain some ^{14}C. Therefore these fractions were subjected to peptide sequencing (15 cycles) and both fractions contained at least one peptide that corresponded to sequences in scpdc1. Fraction 95 contained two clearly identified sequences: MIEVMLPVFDAPQNL, corresponding to residues 535−549, and QVNVNTVFGLPGDFN, corresponding to residues 16−30. Fraction 97 contained one clearly identifiable peptide with the sequence LLQTPIDMSLKPNPA, which matches residues 180−194 in PDC1. There were no unusual amino acids that would signal alkylation of any nucleophilic amino acid detected in these fractions. On the other hand, each fraction has an Asp present in the sequence, and one could envision a Michael addition of the carboxylates to CPB, or the aldehyde product, leading to an adduct (an ester) that may undergo elimination during the peptide sequencing experiments, thereby regenerating unmodified protein. The kinetic studies with the D28A variant (see below) ruled out any role for D28 in the inactivation mechanism. Also, these fractions contain significantly less radioactivity compared to the principal fraction that contains C221.

Determination of the Type of Bond Formed between [3-^{3}H]-CPB and PDC. The tritium content of the fractions collected following HPLC analysis of the tryptic digest of the enzyme reacted with [3-^{3}H]CPB and [1-^{14}C]CPB can be seen in Figure 1C. Following hydroxylamine treatment of fraction 91 (which contains the tritiated peptide encompassing C221), the treated sample was again applied to the C-18 HPLC column and the position of the fraction containing the peak amount of ^{3}H was again monitored (data not shown). Simultaneous treatment of the SLG control with hydroxylamine resulted in 87% hydroxylaminolysis of the thiol ester. HPLC analysis revealed very little change in the position of the ^{3}H peak following treatment by hydroxylamine, which indicates that most of the label is still associated with the peptide and thus *suggests the absence of a thiol ester bond between the tritiated inhibitor and C221*.

Peptide Analysis of the Fractions Containing [3-^{3}H]CPB and the [2-^{14}C]ThDP−PDC Complex. HPLC analysis revealed that ThDP eluted between 10 and 11 min under the chromatographic conditions previously outlined for the semipreparative column. The radioactivity of fractions collected during elution of the tryptic digest of [2-^{14}C]ThDP−PDC that had been reacted with [3-^{3}H]CPB revealed that most of the ^{14}C eluted from the column (approximately 85%) between 10 and 11 min and represents free [2-^{14}C]ThDP (data not shown). The remaining fractions contained no significant amounts of ^{14}C. The most significant result of this HPLC analysis is the *total absence of any fraction containing significant amounts of both ^{3}H and ^{14}C.*

Tryptic digestion of the labeled enzyme followed by peptide sequencing revealed the presence of an unknown doublet at position 221, which would be consistent with a reaction between C221 and cinnamaldehyde via a Michael addition, a common pathway utilized by suicide inhibitors (Fersht, 1985). Such a reaction would produce two diastereomeric

Scheme 1: Proposed Reactions of PDC with (*E*)-2-Oxo-4-phenyl-3-butenoic Acid and Its Decarboxylated Product



thioethers as outlined in Scheme 1 (right-hand column), thus resulting in the doublet obtained following sequencing and amino acid analysis. Uchida and Stadtman (1992) reported the formation of thioethers between protein SH groups and α,β-unsaturated aldehydes. Since hydroxylamine treatment did not release the tritiated inhibitor from the peptide, the presence of a thiol ester bond between the inhibitor and C221 is unlikely. Had C221 formed a thiol ester after decarboxylation of CPB [see possible sources of dihydrocinnamic acids from such compounds in Scheme 1, left-hand column, and Zeng et al., (1991)], the label would have been cleaved on treatment with hydroxylamine and the amount of radioactivity present in the peptide would have been greatly reduced or eliminated. That this was not the case suggests the presence of a thioether bond between C221 and the cinnamaldehyde product of the reaction, which is also consistent with the doublet observed in the sequencing studies.

The stoichiometry of 2 mol of tritiated inhibitor/tetrameric PDC may signal half-of-the-regulatory site activity, consistent with the "dimer-of-dimers" subunit symmetry of PDC suggested by the X-ray results (Dyda et al., 1993; Arjunan et al., 1996).

When dual labels were used ([3-^{3}H]CPB and [1-^{14}C]CPB), there was evidence that in the holoenzyme there was some CPB present (Figure 1C). Sequencing of HPLC fractions containing ^{14}C activity after reaction of the enzyme with both [3-^{3}H]CPB and [1-^{14}C]CPB did not reveal the presence of any unusual amino acids, i.e., residues containing modified side chains. The peptide sequences in fractions 95 and 97 also reveal that they are not part of the ThDP-binding sequence (Dyda et al., 1993; Arjunan et al., 1996). Hence the presence of ^{14}C in fractions 95 and 97 can be attributed to one or both of the following possibilities: (1) residual free [1-^{14}C]CPB or (2) nominal reaction of [1-^{14}C]-CPB at a residue other than cysteine (see below). The very substantial amount of ^{3}H found in fractions 100−101 in Figure 1C, and to a lesser extent in Figure 1A, was attributed to undigested labeled PDC, i.e., the intact enzyme eluted at this position in this solvent system (data not shown). There was, however, *no ^{14}C detected in the peptide encompassing C221* (i.e., fraction 91), and had CPB itself formed a covalent linkage with the enzyme, that bond did not survive the sequencing protocol.

Earlier work by this laboratory had shown that the enamine

formed between these 2-oxo acids and ThDP−PDC is relatively long-lived (Kuo & Jordan, 1983b; Kuo et al., 1986; Zeng et al., 1991; Menon-Rudolph et al., 1992), raising the possibility (Jordan et al., 1988) that a ThDP−CPB complex may form a ternary complex with PDC. There is precedent for the formation of such a ternary complex among D-amino acid transaminase, its cofactor pyridoxal 5′-phosphate, and a substrate, D-alanine (Martinez del Pozo et al., 1992). The lack of simultaneous presence of *both* ^{3}H and ^{14}C in any single fraction of the HPLC analysis of the tryptic digest of [2-^{14}C]ThDP−PDC that reacted with [3-^{3}H]CPB appears to rule out a covalent interaction between an enamine-like complex and a residue on the enzyme. However, the results cannot rule out the possibility that such a ThDP−CPB complex is responsible for inactivation of the enzyme and that such a complex would be released from the enzyme during the trypsinization.

The most important protein chemical finding of this study is that the *cinnamaldehyde product, but not CPB itself, has been shown to be covalently bonded to C221*, further corroborating the regulatory role and high reactivity of C221 in PDC activity. Early kinetic studies of the interaction of PDC with CPB and related compounds implicated reactions at both the active site and the regulatory site (Kuo & Jordan, 1983a), while more recent work with a fusion enzyme containing a single cysteine at position 221 (Zeng et al., 1993) and the work with the scpdc1 cysteine variants (Baburina et al., 1994, 1996) unambiguously assigned a regulatory role to C221. The X-ray studies (Dyda et al., 1993; Arjunan et al., 1996) revealed that C221 is located >20 Å from the ThDP-binding site, a distance that surely would obviate a role for this residue at the active site. However, the ability of C221 to play a regulatory role at that distance has a precedent, for example, in the allosteric inhibition of *E. coli* citrate synthase (Donald et al., 1991). C206 of that enzyme has a cysteine residue at position 206, which is 25−30 Å from the active site and, like C221 of PDC, lies at the surface of a subunit. The inhibitor NADH was found to bind at or very close to C206, thus demonstrating that C206 can, even at a distance of 25−30 Å, communicate with and influence the active site.

Inhibition Kinetics of WT PDC and Variants by CPB, NPB, and NA. Once protein chemical studies identified the cinnamaldehyde product being bonded to C221 rather than the 2-oxo acid precursor, we wished to answer several questions: (1) Are variants at C221 still subject to inactivation by CPB and its product? (2) What are the relative inhibitory efficacies of CPB-like compounds compared to the corresponding decarboxylated cinnamaldehyde product? (3) Can other cysteine residues be modified by CPB or its product and thereby inactivate PDC? (4) Could the serine residue in the C221S variant act as a nucleophile and interact with CPB or its product? (5) Can other potential nucleophilic sites at either the regulatory or catalytic site interact with CPB? (6) Does the C221S variant lacking the regulatory site possess some features that make it more susceptible to inactivation by CPB-like reagents, perhaps with a mechanism different from that experienced by WT PDC?

In response to the first question, it was surprising to find that the C221S PDC variant, as well as the C222S and C221S/C222S variants, is also inactivated by CPB within the same time and nearly to the same extent as WT PDC (Figure 2). Neither hydroxylamine treatment nor dilution followed by dialysis of the inactivated enzyme rescued the activity. However, these attempts to recover activity are inconclusive since PDC variants that had not been inactivated also tended to lose activity under these conditions. Addition of excess ThDP to the cysteine variants, before or after inactivation, did not influence the extent or reversibility of inactivation, indicating that the ThDP did not dissociate from the enzyme under the influence of the inhibitor.

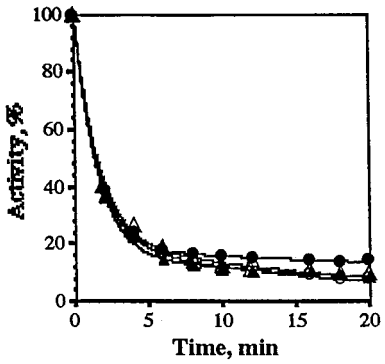


FIGURE 2: Inactivation of WT PDC (○), C221S PDC (●), C222S PDC (△), and C221S/C222S PDC (▲) with CPB. The incubation mixture contained 30 units of PDC in 0.1 M MES, pH 6.0, 1 mM ThDP, 2 mM Mg^{2+}, 1 mM EDTA, and 2 mM CPB.

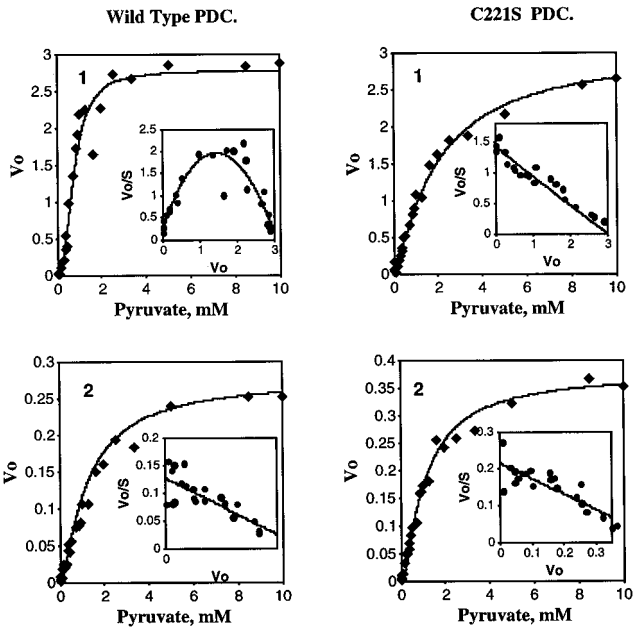


FIGURE 3: v_0 vs S plots before and after inactivation with CPB. (1, top panels) WT and C221S PDC. (2, bottom panels) After reaction with 1 mM CPB for 20 min. Residual activity was approximately 7% for each experiment. Insets: Eadie−Scatchard plots.

WT PDC was inactivated with 2 mM CPB for 30 min to 7% of its initial activity and then assayed (Figure 3). WT PDC in the absence of inhibitor gives the typical v_0 vs S plot with a Hill coefficient $n_H = 1.89$, $K'_m = 0.82$ mM. PDC inactivated by CPB exhibits hyperbolic kinetics with $K'_m = 1.8$, $n_H = 0.98$. This behavior is consistent with the loss of regulatory properties due to modification of C221. Similar kinetic studies on the C221S variant give a $K_m = 5.6$ mM and $n_H = 1.0$ for the enzyme before and after inactivation with CPB, although the activity drops to 7.5% of its value prior to inhibition, i.e., by nearly the same fraction as it does

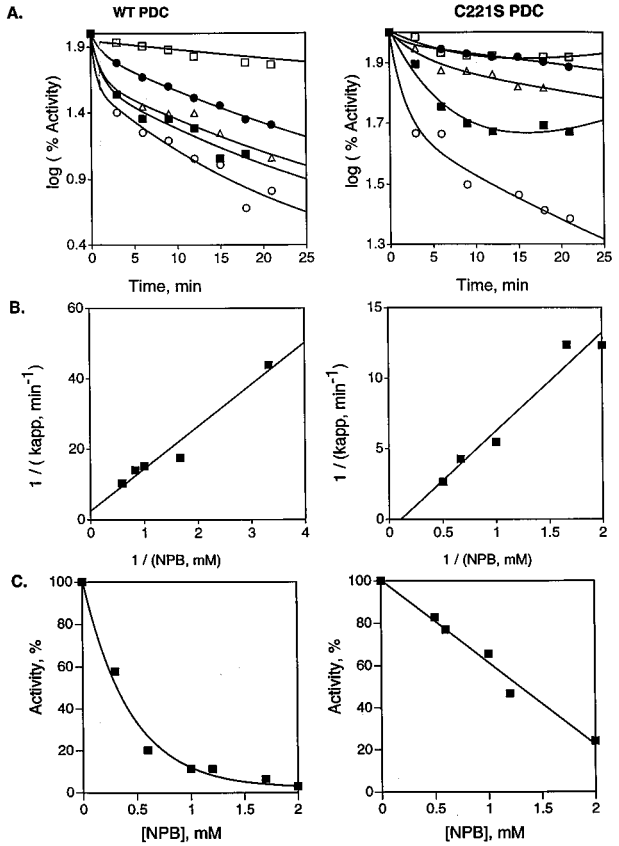


FIGURE 4: Inactivation of WT PDC and C221S PDC with NPB. (A) Time-course of inactivation of 30 units of WT PDC (left side) and 30 units of C221S PDC (right side) with (from top to bottom) 0.3, 0.6, 1.0, 1.2, and 1.7 mM NPB for WT PDC and 0.5, 0.6, 1.0, 1.2, and 2 mM NPB for C221S PDC. (B) Kitz−Wilson plots. (C) Analysis for multisite interaction of PDC with NPB plotting activity remaining after 25 min.

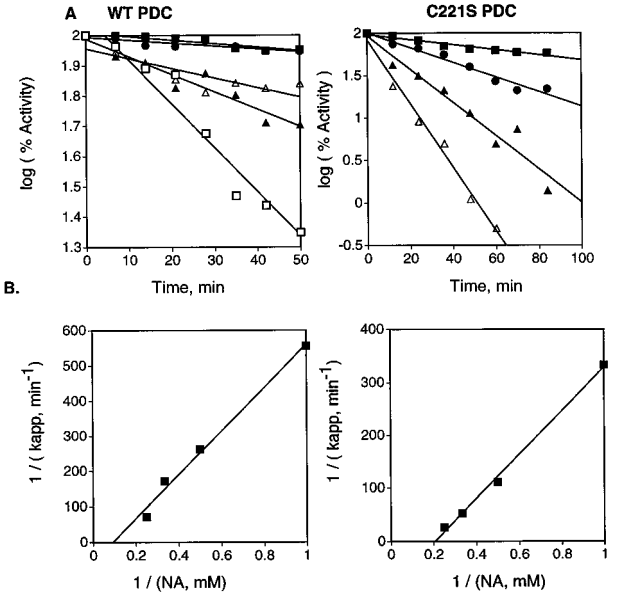


FIGURE 5: Inactivation of WT PDC and C221S PDC with NA. (A) Semilog plots of the time course of inactivation of 30 units of WT PDC (left side) and 30 units of C221S PDC (right side) with (from top to bottom) 1, 2, 3, and 4 mM NA. (B) Kitz−Wilson plots.

for the WT PDC on treatment with CPB. It is important to recall, however, that the specific activity of the C221S variant is only 20−30% that of the WT PDC [see Baburina et al. (1998)]. Additional experiments showed that C222 does not participate in these reactions, as the C221S/C222S doubly substituted PDC behaved as the C221S variant with respect to CPB, while the C222S variant behaves very similarly to the WT PDC.

To address the second question, we compared the relative inhibitory efficacy toward WT and C221S PDC of (*E*)-(4-nitrophenyl)-2-oxo-3-butenoic acid (NPB) and the product of its decarboxylation, 4-nitrocinnamaldehyde (NA). The time-course of inactivation with NPB (Figure 4) does not obey a first-order rate law (log activity vs time plots are not linear). The Kitz−Wilson plots suggest that the inhibitor forms a reversible complex with WT PDC, but not with the C221S variant, prior to irreversible inhibition. Analysis of the percent activity vs [I] plots shows multiple site inhibition of WT PDC by NPB, or depletion of NPB due to concomitant turnover, and single-site inhibition of C221S PDC by NPB. The semilog plots of percent activity vs time for both WT and C221S PDC with NA are linear (Figure 5), indicating that the reaction obeys first-order kinetics. Kitz−Wilson plots show neither WT PDC nor the C221S variant being saturated with NA. Inhibition by NA of both WT and C221S is rather slow, with an estimated $t_{1/2}$ for low concentrations of inhibitor as long as 600 min; hence no accurate determination of the reaction order was possible.

The second-order rate constants for inactivation by NPB are estimated at 83.7 (WT) and 143 M^{-1} min^{-1} (C221S), and for inactivation by NA, at 1.62 (WT) and 2.41 M^{-1} min^{-1} (C221S), indicating that NPB is approximately 50−60 times more potent than NA, and both NPB and NA are approximately 1.5 times more reactive with the C221S variant than with the WT PDC. Apparently, PDC that is lacking the regulatory mechanism is more vulnerable to active-site-directed inhibition by both compounds, providing a partial answer to question 6.

It was unexpected that NPB and NA could also inhibit the C221S variant, clearly showing that there is at *least one site, in addition to C221, at which these compounds can inactivate PDC*. The finding that inactivation by NPB appears to take place at multiple sites on the WT PDC and only at one site on the C221S variant is consistent with reaction at the second site (probably ThDP) in both enzymes and additionally at C221 in the WT PDC.

The study by Zeng et al. (1991) had already hinted at inhibition of PDC by the aldehyde product, since in the presence of the allosteric modifier pyruvamide the molar ratio of *p*-methylcinnamaldehyde:*p*-methyldihydrocinnamic acid had increased, resulting in faster inhibition by *p*-$CH_3C_6H_4$-CH═CHCOCOOH (TB). The kinetic data clearly show that C221 is indeed modified concomitant with inactivation, since the v_0 vs [S] plot of the CPB-modified WT PDC is hyperbolic [as is the plot for the C221S variant; see Baburina et al. (1994, 1998)], whereas that of the WT PDC is sigmoidal.

Pyruvamide [a substrate surrogate that cannot be decarboxylated but activates PDC; see Hubner et al. (19780 and Baburina et al. (1998)] at 50 mM concentration affords modest protection (10−15%) from NPB, while it offers no protection from inhibition by NA (data not shown). Pyruvamide has no effect on the inhibition of C221S PDC by any of the three reagents. This result provides further

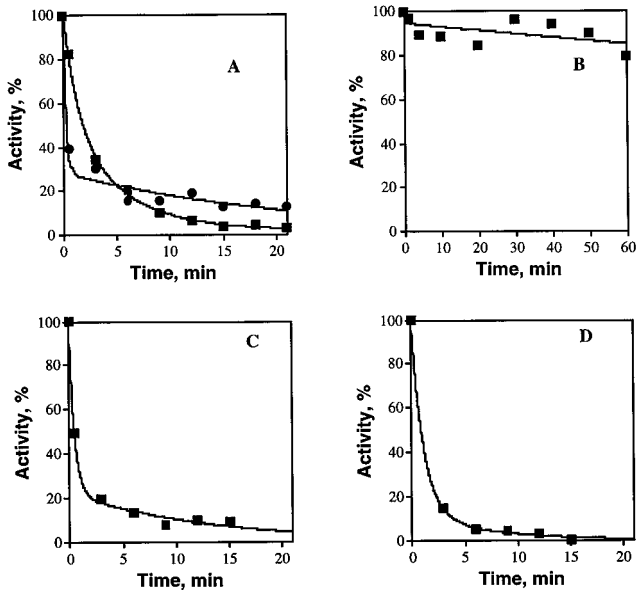


FIGURE 6: Inactivation of C152A, C222A, and C221A/C222A variants with CPB, NPB, or NA. (A) C152A at 2 mM CPB (■) or 2 mM NPB (●). (B) C152A at 2 mM NA. (C) C222A at 2 mM CPB. (D) C221A/C222A at 2 mM CPB.

definitive evidence for pyruvamide reacting at C221 with the WT PDC.

To address the third and fourth questions (whether the third accessible cysteine at position 152 or the serine in place of cysteine at positions 221 and 222 would also react with CPB), inactivation of the C152A and C221A/C222A variants with CPB, NPB, and NA was studied. Inactivation plots for the C152A, C222A and C221A/C222A variants (Figure 6) exhibit inhibition patterns similar to those by the WT PDC when exposed to the same reagent (compare with Figure 2). An exception perhaps is the observation that the C152A variant suffers virtually no inactivation by NA, raising the possibility that C152 in the WT PDC does react with high concentrations of NA, albeit at a slow rate. Since the C222A and C221A/C222A variants of PDC are also inhibited by CPB, we can rule out interaction of CPB with the serine 221 in the C221S variant. While the C221S PDC variant has only 20−30% the specific activity of the WT PDC, the C222S and C152A variants are quite active and are both inhibited to the same extent as WT PDC by CPB. At the same time, C69, the fourth cysteine, is buried deep inside the protein. These experiments lead to the conclusion that *C221 is by far the most reactive cysteine in PDC*.

The fifth question was addressed by studying the inactivation of the D28A, E477Q, E477D, H114F, and H92K variants with CPB, NPB, and NA. The first three residues are in the active center and their variants have the potential to identify active-center interaction with the inhibitors CPB, NPB, and NA. While the D28A and H114F variants are inactivated faster than WT PDC, the E477Q and E477D variants were inactivated much faster by all three compounds under the same conditions (Guo, Wang, and Jordan, manuscript in preparation). The H92K variant [see Baburina et al. (1998)] was tested to determine whether H92, a participant in the substrate activation cascade, is perhaps the active nucleophile. In fact, the H92K variant is inactivated at nearly the same rate as WT PDC. This is consistent with the notion that H92 (or H92K) is protonated at pH 6.0, the optimum pH for this enzyme, and a protonated histidine would not be nucleophilic.

The last question has only been partially resolved since no protein chemical evidence was obtained for a covalent bond between PDC and CPB itself; rather, only with the cinnamaldehyde product. What is apparent already is that both the CPB and its aldehyde product (see section with NPB and NA) react somewhat faster with the PDC with an impaired regulatory site (C221S) than with the WT PDC. This is also observed in studies with glyoxalic acid (see below and Figure S8 in the supporting information). The structural basis for this is not yet evident from the existing X-ray structures.

The finding that the D28A, H114F, H115F, and E477Q variants, with substitution at the active center, are also inactivated by CPB, *even faster than WT PDC*, suggests that ThDP in the active center is involved. When both labels are used, the stoichiometry of [^{3}H] to [^{14}C] is approximately (2−3):1 with holo-PDC following inhibition, consistent with binding of 1 CPB (carrying both labels) and 1 or 2 aldehyde product molecules (carrying only the tritium label) in the intact PDC, perhaps the aldehyde at C221 and CPB at the active center. Reaction of CPB at ThDP would be very difficult to detect, since there was no evidence obtained for formation of a ternary PDC−ThDP−CPB complex (data not shown). Any experiment that would lead to destruction of tertiary and quaternary structure would extrude from the protein a CPB−ThDP adduct. Indeed, there is chemical precedent for interaction of ThDP with aldehydes leading to benzoin products (Chen & Jordan, 1984), a slow and inefficient reaction, as is the inhibition by NA. In addition, the conjugated 2-oxo acids, such as CPB, undergo substrate-like turnover as well. The observation that WT PDC and the C221S variant undergo approximately the same fractional (%) inactivation under similar conditions, even though the C221S variant is only 20−30% as active as the WT PDC, suggests a common additional mechanism of inactivation, likely at ThDP.

With our current state of knowledge regarding PDC inactivation by conjugated substrate analogues in this class of inhibitors we conclude the following:

(1) C221 is modified concomitant with inactivation, but this modification need not lead to total loss of activity. On the other hand, we have also observed that several alkylating agents, iodoacetate, 1-bromo-2-butanone, and 1,3-dibromo-2-propanone, also inactivate (at least partially) PDC, presumably by reacting at C221, since the substrate activation is also abolished [see Baburina et al. (1998)]. It is also relevant to note that 8-bromoacetyl-10-methylisoalloxazine (in a model for the enzyme pyruvate oxidase) virtually completely inactivated PDC, presumably by reaction at C221 (Annan & Jordan, 1990).

(2) Both WT PDC and its regulatory site C221S variant are still subject to inactivation, signaling an inactivation site in addition to C221.

(3) Since the C221S variant has only 20−30% residual activity, CPB or NPB attached to C221 in the WT PDC could produce the same magnitude of reduction in activity, thereby accounting for most of the inactivation of WT PDC. In fact, some CPB analogues, such as those with *p*-CF_3 and *m*-CF_3, completely inactivate PDC (Annan et al., 1989).

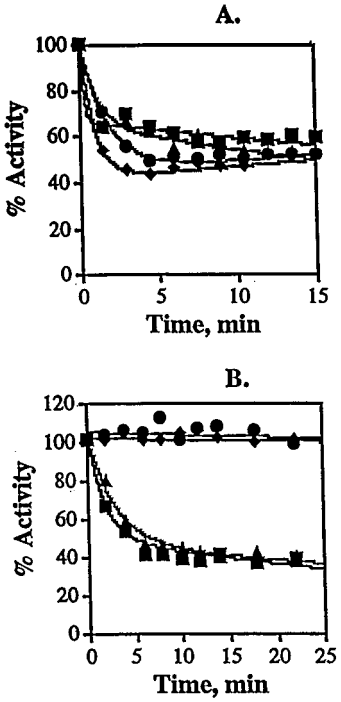


FIGURE 7: Inactivation of PDC variants by glyoxalic acid and ketomalonate. (A, 1 mM glyoxalic acid) WT PDC (●), C221S PDC (◆), C222S PDC (■), and C221S/C222S PDC (▲). (B, 1 mM ketomalonate) WT PDC (■), C221S PDC (◆), C222S PDC (▲), and C221S/C222S PDC (●).

(4) The decarboxylation product of NPB (NA) also interacts with PDC at a site in addition to C221, a site modified by the decarboxylated product of CPB, as demonstrated by the labeling experiments. Since NPB is a much more effective inhibitor than NA, NPB must be the predominant inactivator during the short time course of inactivation.

(5) The inactivation of the D28A, E477Q, E477D, H114F, and H92K variants with CPB *directly rules out essentially all but C221 and ThDP as potential reactive groups vis-à-vis CPB at both the catalytic and the regulatory sites.*

(B) Interactions of WT and PDC Variants with Glyoxalic Acid, Ketomalonate, and Traditional Sulfhydryl-Specific Reagents

Glyoxalic Acid and Ketomalonate. Over the years, there has been considerable evidence presented that glyoxalic acid is a mechanism-based inactivator (Uhlemann & Schellenberger, 1976), while ketomalonate is a "lock-on" activator of PDC (Alvarez & Schowen, 1991). The PDC variants provide an opportunity to identify the site specificity of these compounds.

Glyoxalic acid provides very similar inhibition profiles with both WT PDC and C221S PDC (Figure 7), with the formation of a reversible enzyme-inhibitor complex (Figure S8), in agreement with the mechanism proposed by Uhlemann and Schellenberger (1976), which called for decarboxylation. The plots for (% A) vs [I] are different for the WT (multiple-site inhibition) and C221S variant (single-site inhibition) PDCs, perhaps signaling that at higher concentrations glyoxalic acid also occupies the regulatory site, thereby augmenting the inhibition. The Hill coefficient of WT PDC inactivated by 2 mM glyoxalic acid decreases to 1.4 from 2.0 for unmodified enzyme (Figure S10), also consistent with additional interaction at C221.

Table 1: Kinetic Properties of WT PDC Inhibited with Different Concentrations of Ketomalonate

ketomalonate (mM)	% activity remaining	n_H[a]
0	100	2
2	22	1.53
10	3	1.29
25	2.8	1.21
50	2.7	0.99

[a] Hill coefficient from the fit of the kinetic data in Figure S10 to a sigmoidal curve, $R^2 > 0.99$.

The C221S and C221S/C222S PDC variants are not affected by ketomalonate, but both the WT and the C222S variant are inactivated similarly (Figure 7). More extensive kinetic results reinforce the inactivity of the C221S PDC vis-à-vis ketomalonate (Figure S9D), also showing that the WT PDC does not form a noncovalent complex prior to inhibition according to the Kitz-Wilson plot (Figure S9 B). Steady-state kinetic data suggest that the inhibition by ketomalonate is reversible (see below). Michaelis−Menten plots for WT PDC first inhibited with increasing concentrations of ketomalonate show a decrease of the Hill coefficient with increasing concentration of ketomalonate (Figure S10 and Table 1).

The kinetic data with the C221S variant (Figure S9D) unambiguously show that ketomalonate interacts with C221, the site of substrate activation, supporting the report that ketomalonate is an activator of PDC (Alvarez & Schowen, 1991). It is to be noted though that the v_0 vs S plots assume fully hyperbolic behavior only at rather high (25 mM) concentrations of ketomalonate, which could be due to the reversibility of the reaction in the presence of pyruvate at such high concentrations. The reversibility is supported by the finding that the Hill coefficient decreases further with additional ketomalonate in the inhibition mix. The (% A) vs [I] plot shows the possibility of multiple-site inhibition and could be the result of occupation of the active site by ketomalonate at high concentrations.

Reaction of WT and Variant PDCs with pCMB and DTNB. In the accompanying paper (Baburina et al., 1998), it is shown by DTNB titration that three of four cysteines are accessible in WT and two of three in the C221S variant of PDC. C69 is buried deep inside the protein. Both WT PDC and the C221S variant are inactivated in a time-dependent fashion by 1 mM pCMB within 30 s, while inactivation by DTNB is slower and less effective (Figure S11). The effect of both *p*CMB and DTNB is reversed by DTT. The time course of reaction between different PDC variants and *p*CMB and DTNB is not significantly different (data not shown).

Inactivation of PDC and of all the variants with *p*CMB is much faster and more complete than with DTNB. It is possible that the different pattern of PDC inactivation exhibited by pCMB and DTNB is a consequence of their structure: the introduction of a bulky residue into the protein molecule causes the loss of tertiary interactions. The nearly quantitative restoration of the activity by DTT rules out any reaction other than at a thiol.

CONCLUSIONS

How do we reconcile the fact that the C221S variant is 20−30% as active as the WT PDC, while anything added

to C221 in WT PDC may reduce the activity even further, in some cases to essentially zero within experimental error? Model building has shown that anything larger than pyruvate attached to C221 in the hemithioketal form exerts a mechanical force that pushes the α and β domains further apart. This perturbation must be propagated throughout these domains and may also result in distortions at the active center. A model that is consistent with all of the data accumulated so far would have several states of the enzyme: (1) In the presence of pyruvate only at the pH optimum, there is strong positive cooperativity with $n_H \sim 2.0$ and K_m' near 1 mM. (2) In the presence of saturating pyruvamide, the K_m is excellent (~1 mM) but $n_H \sim 1.0$; hence this enzyme is fully activated. (3) In the C221S (or C221A/C222A) variant, or in the H92G and H92A variants, the K_m is substantially increased and n_H tends to unity; this form of PDC is no longer subject to substrate activation (see data with pyruvamide). (4) *Any* group other than pyruvate or pyruvamide attached to C221 very strongly reduces the activity while abolishing cooperativity as well. Apparently, the interaction between domains is very delicately balanced and too large a steric bulk at the domain interface can lead to distortion of the active center as well as to abolition of cooperativity. Whether these distortions simply hinder access to the active center or compromise optimal reaction of active-center residues with the substrate during turnover will have to await further structural studies. The observation that the reactivities of WT and C221S PDC are similar vis-à-vis CPB or NPB or NA, and the results ruling out reaction with virtually any other potential nucleophile, suggest exclusive reactivity at these C221 and ThDP, consistent with space-filling models of PDC [Arjunan et al., 1996] showing that the reactive site of ThDP and C221 are *the only surface-accessible loci in the structure.*

This work further underscores the remarkable reactivity of C221 and its role in regulation of yeast pyruvate decarboxylase and thus clearly corroborates the recent work of Zeng et al. (1993) and Baburina et al. (1994, 1996). It is totally consistent with the high reactivity predicted for the thiolate form adjacent to a histidinium residue. In addition, this study also substantiates the accumulated studies of other workers over the past 50 years, studies that have implicated a role for a cysteine residue in yeast PDC activity.

SUPPORTING INFORMATION AVAILABLE

Description of the synthesis of [2-^{14}C]ThDP, [3-^{3}H]CPB, and [1-^{14}C]CPB and Figures S8−S11, showing PDC interaction with glyoxalic acid, ketomalonate, *p*CMB, and DTNB (7 pages). Ordering information is given on any current masthead page.

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