Role of Cysteines in the Activation and Inactivation of Brewers' Yeast Pyruvate Decarboxylase Investigated with a *PDC1–PDC6* Fusion Protein†

Xiaoping Zeng,‡ Bruce Farrenkopf,‡ Stefan Hohmann,§ Fred Dyda,∥ William Furey,∥ and Frank Jordan*,‡

Department of Chemistry, Rutgers, the State University, Newark, New Jersey 07102, Laboratorium voor Moleculaire Celbiologie, Instituut voor Plantkunde, Katholieke Universiteit te Leuven, Kardinaal Mercierlaan 92, B-3001 Leuven-Heverlee, Flanders, Belgium, and Biocrystallography Laboratory, Veterans Administration Medical Center, P.O. Box 12055, University Drive C, Pittsburgh, Pennsylvania 15240

Received May 7, 1992; Revised Manuscript Received November 30, 1992

ABSTRACT: Possible roles of the Cys side chains in the activation and inactivation mechanisms of brewers' yeast pyruvate decarboxylase were investigated by comparing the behavior of the tetrameric enzyme pdc1 containing four cysteines/subunit (positions 69, 152, 221, and 222) with that of a fusion enzyme (pdc1–6, a result of spontaneous gene fusion between *PDC1* and *PDC6* genes) that is 84% identical in sequence with pdc1 and has only Cys221 (the other three Cys being replaced by aliphatic side chains). The two forms of the enzyme are rather similar so far as steady-state kinetic parameters and substrate activation are considered, as tested for activation by the substrate surrogate pyruvamide. Therefore, if a cysteine is responsible for substrate activation, it must be Cys221. The inactivation of the two enzymes was tested with several inhibitors. Methylmethanethiol sulfonate, a broad spectrum sulfhydryl reagent, could substantially inactivate both enzymes, but was slightly less effective toward the fusion enzyme. (*p*-Nitrobenzoyl)formic acid is an excellent alternate substrate, whose decarboxylation product *p*-nitrobenzaldehyde inhibited both enzymes possibly at a Cys221, the only one still present in the fusion enzyme. Exposure of the fusion enzyme, just as of pdc1, to (*E*)-2-oxo-4-phenyl-3-butenoic acid type inhibitors/alternate substrates enabled detection of the enzyme-bound enamine intermediate at 440 nm. However, unlike pdc1, the fusion enzyme was not irreversibly inactivated by these substrates. These substrates are now known to cause inactivation of pdc1 with concomitant modification of one Cys of the four [Zeng, X.; Chung, A.; Haran, M.; Jordan, F. (1991) *J. Am. Chem. Soc. 113,* 5842–49]. Of the two adjacent cysteines, Cys221 and Cys222, at least Cys221, and perhaps even Cys222, is likely to be important for fully functioning native pyruvate decarboxylase from brewers' yeast.

The simplest nonoxidative decarboxylation of α-keto acids is performed by pyruvate decarboxylase (PDC,[1] EC 4.1.1.1). As is the case with nearly all such enzymes, thiamin diphosphate (ThDP) is an essential cofactor (see reviews in Krampitz (1969, 1970), Sable and Gubler (1982), Kluger (1987), Schellenberger and Schowen (1988), and Bisswanger and Ullrich (1991)). While the enzyme is capable of accelerating the reaction over the nonenzymic, i.e., ThDP-catalyzed, model reaction by a factor of 10^{12} (Alvarez et al., 1991), chemical modification studies have not revealed the participation of any potential general acid–base or nucleophilic enzymic side chain in the reaction mechanism, except possibly for Cys (Ullrich, 1982). That Cys modification greatly reduces the activity has been reported repeatedly for nearly 50 years (see Jordan et al. (1988) for a detailed reference list). It has also been known for some time that the enzyme isolated from brewers' yeast is subject to substrate activation (Boiteux & Hess, 1970; Hübner et al., 1978), an activation that can be mimicked by pyruvamide, a nondecarboxylatable pyruvate surrogate. It has been hypothesized that a Cys is present at the substrate activation site (Sieber et al., 1983; Schellenberger et al., 1988), supported by the irreversible inactivation of the enzyme by 3-bromopyruvamide, an electrophilic analogue of pyruvamide (Hübner et al., 1978). Alternatively, a Cys may be important in product release (Jordan et al., 1988). This hypothesis was based on the observation that methylmethanethiol sulfonate (MMTS) inactivated PDC could still develop the visible absorbance with a maximum near 440 nm that is characteristic of the enamine intermediate (Scheme I) derived from complexes with (*E*)-2-oxo-4-phenyl-3-butenoic acid type inhibitors/alternate substrates (Kuo & Jordan, 1983; Jordan et al., 1986, 1988). Recently, Zeng et al. (1991) reported that inactivation by one such inhibitor, (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid (TB), was accompanied by the modification of one of four Cys/subunit, strongly implying that whether or not a Cys side chain directly participates in catalysis, there is one Cys whose modification with such large electrophilic compounds greatly inhibits the reactivity of PDC (Scheme I).

During the past decade the PDC protein sequence from a variety of sources has been deduced from the DNA sequence. In the yeast *Saccharomyces cerevisiae*, PDC appears to be a mixture of the products of the genes *PDC1* (the corresponding enzyme henceforth scpdc1) and *PDC5*. The two isozymes are 88% identical and both have four conserved Cys at positions

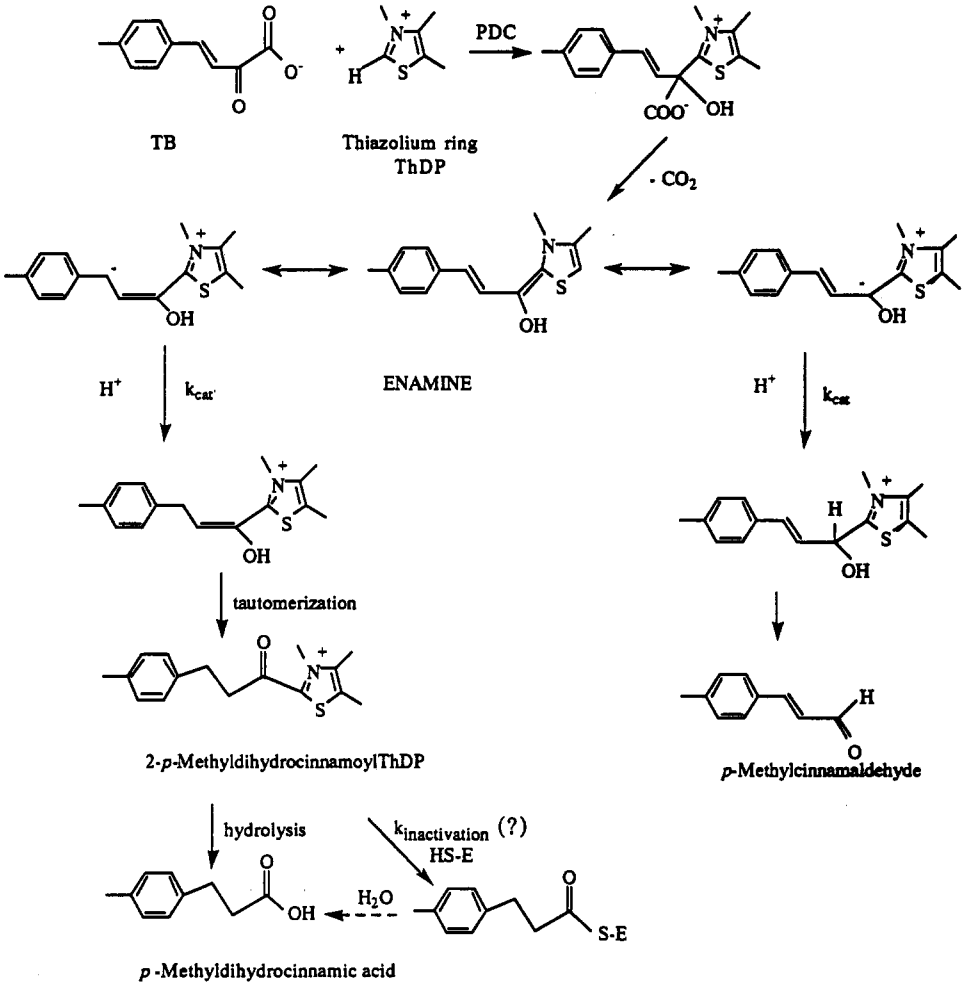
† Supported at Rutgers (F.J.) by NSF-DMB, the Rutgers Busch Biomedical Fund and Hoffmann-La Roche, Inc., Nutley, NJ, and at Pittsburgh (W.F.) by NIH-GM48195-01.

* Author to whom correspondence should be addressed.
‡ Rutgers, the State University.
§ Katholieke Universiteit te Leuven.
∥ Veterans Administration Medical Center.

[1] Abbreviations used: ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase (E.C. 4.1.1.1); supdc1, pyruvate decarboxylase isolated from *Saccharomyces uvarum*; scpdc1, pyruvate decarboxylase isolated from *Saccharomyces cerevisiae*; scpdc1–6, pyruvate decarboxylase isolated from the spontaneous fusion gene *Saccharomyces cerevisiae PDC1–PDC6*; CPB, (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid; TB, (*E*)-2-oxo-4-tolyl-3-butenoic acid; MMTS, methylmethanethiol sulfonate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/93/0432-2704$04.00/0

Scheme I



69, 152, 221, and 222 (Hohmann & Cederberg, 1990). According to Zeng et al. (1991), all four cysteines are titratable in native wild-type pyruvate decarboxylase, i.e., there is no evidence for disulfides in the structure. A third structural gene in yeast is *PDC6*. This gene appears to be expressed at only very low levels, and thus it does not contribute appreciably to the PDC activity (Hohmann, 1991a). Remarkably, the product of *PDC6* as deduced from the DNA sequence contains only one Cys at position 221, while the other three Cys residues are replaced by aliphatic amino acids. Using molecular genetic techniques and, in addition, taking advantage of a spontaneous recombination event betwen *PDC1* and *PDC6*, a yeast strain has been constructed which contains as its sole source for PDC a fusion gene consisting of the 45 N-terminal amino acids of *PDC1* and the 520 C-terminal amino acids of *PDC6* (Hohmann, 1991b,c). The length of this fusion gene product is the same as that of the wild-type enzymes, but the product of this fusion gene (henceforth scpdc1–6) has only the single Cys at position 221. We have undertaken a comparison of the kinetic properties of the fusion protein with *PDC1* from two yeast strains: *Saccharomyces cerevisiae* (scpdc1) and *Saccharomyces uvarum* [the latter supdc1 has been studied for two decades by the Rutgers group, and one of its isozymes has been subjected to X-ray crystallographic analysis (Dyda et al., 1990, 1991; Dyda, 1992)]. The experiments provide insight concerning which Cys may have a role in the functioning of the enzyme.

EXPERIMENTAL SECTION

Yeast Strain Used as Source of Enzyme. The yeast strain expressing the scpdc1–6 fusion protein has been described by Hohmann (1991b).

Enzyme Purification and Assay. The enzyme was purified according to Farrenkopf's protocol (Farrenkopf & Jordan, 1992), with an improved resolution over the procedure of Kuo et al. (1986). Protein assay was performed by the Bradford (1976) method, and the enzyme was assayed by the pH–stat method (Schellenberger et al., 1968) or the aldehyde dehydrogenase coupled assay (Holzer et al., 1956). One unit of activity is defined as the amount of PDC required to convert 1 μmol of pyruvate to acetaldehyde per minute at 30 °C at pH 6.0.

SDS Gel Electrophoresis of scpdc1–6. Polyacrylamide gel electrophoresis was run under denaturing conditions in the presence of sodium dodecyl sulfate (SDS-PAGE) according to the protocol of Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R.

Time-dependent inactivation studies of scpdc1, supdc1, and scpdc1–6 used methylmethanethiol sulfonate (MMTS), (*p*-nitrobenzoyl)formic acid, (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid (CPB), and (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid (TB). In a typical experiment, 20 units of scpdc1, supdc1, or scpdc1–6 was incubated in 1.0 mL of 0.1 M citrate, pH 6.6, containing 1.0 mM each of ThDP, $MgCl_2$, and EDTA, with 20% ethylene glycol at 25 °C and the concentrations of inhibitor indicated in the figure legends. At the indicated

Table I: Steps in the Purification of the pdc1–6 Fusion Protein

step	total units	specific activity (units/mg)	yield (%)
1. crude extract	4060	0.95	100
2. $(NH_4)_2SO_4$ fractionation	3550	2.92	87
3. heat treatment	2120	5.00	52
4. HTP	650	10.4	16
5. DEAE-5PW HPLC	165	15.8	8[a]

[a] Only 325 units of enzyme was loaded on DEAE-5PW HPLC.

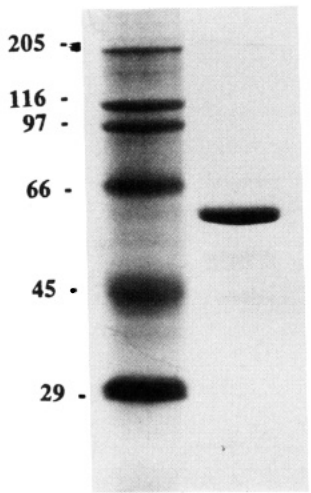


FIGURE 1: SDS-PAGE of scpdc1–6 fusion protein after DEAE-5PW HPLC: lane 1 (left), standard proteins; lane 2 (right), the purified scpdc1–6 fusion protein.

times, aliquots were removed for activity assay with the pH–stat.

RESULTS

Enzyme Purification. Table I summarizes the steps in the purification of scpdc1–6. Figure 1 (right) shows the purity of the scpdc1–6 according to SDS-PAGE. The specific activity of the scpdc1–6 enzyme compared to that of scpdc1 or supdc1 is ca. 30%, whereas the two pdc1 variants have nearly identical specific activities within experimental error. The pH optimum of the fusion protein is shifted to the alkaline side by approximately 0.5–0.6 unit when compared with the scpdc1 and supdc1 enzymes (Figure 2).

Steady-State Kinetic Behavior. As shown in Table II, at saturating pyruvamide (40 mM, under these conditions hyperbolic v_o vs. [substrate] behavior is observed), the k_{cat}/subunit is diminished in scpdc1–6 compared to supdc1. In the absence of pyruvamide, the Hill coefficients are 1.81 and 1.91 for scpdc1–6 and supdc1, respectively.

Time-Dependent Inactivation. (*p*-Nitrobenzoyl)formic acid, methylmethanethiol sulfonate (MMTS), (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid (CPB), and (*E*)-2-oxo-4-*p*-tolyl-3-butenoic acid (TB) were tested as potential inactivators of the scpdc1–6 fusion protein. Figure 3 shows the inactivation of supdc1 and scpdc1–6 by the nonspecific sulfhydryl blocking reagent MMTS. (*p*-Nitrobenzoyl)formic acid caused time-dependent inactivation of all three enzymes (both pdc1s and scpdc1–6), yielding similar inactivation patterns and virtually superimposable ones for the two strains of pdc1 (Figure 4). In Figures 5 and 6 the time courses of inactivation of the enzymes by CPB and TB are shown. While supdc1 is irreversibly inactivated by both compounds, it is clear that scpdc1–6 is only temporarily, and to a much lesser extent, inhibited with full activity being regained in a relatively short time span.

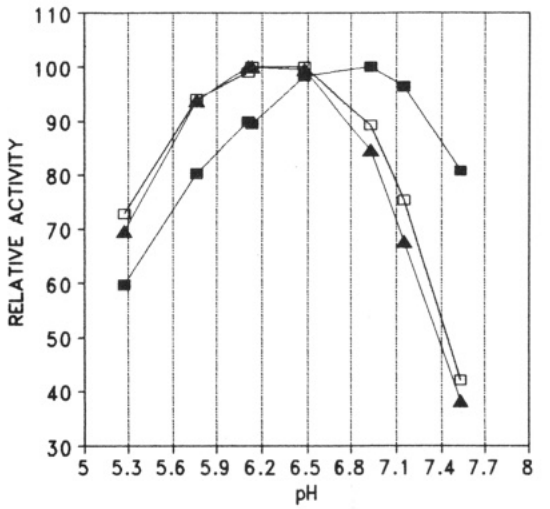


FIGURE 2: Activity–pH profile of scpdc1, supdc1, and scpdc1–6 at 25 °C. Each assay mixture contained 0.73 IU/mL of scpdc1 (□), scpdc1–6 (■), or supdc1 (△), along with 25 IU/mL alcohol dehydrogenase (400 IU/mg), 0.45 mM NADH, and 45 mM pyruvate in 0.1 M MES at the indicated pH values.

Table II: K_m, k_{cat}, and k_{cat}/K_m of supdc1 and scpdc1–6[a]

	pH	K_m (mM)	k_{cat}/active site (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
supdc1	6.0	1.01	62	61
scpdc1–6	6.2	0.80	19	24
scpdc1–6	6.8	6.47	22	3.4

[a] 2 μM supdc1 (specific activity 50 units/mL at 25 °C) or scpdc1–6 (specific activity 15 units/mL at 25 °C) was incubated at 25 °C with different concentrations of pyruvate in 0.1 M MES buffer containing 40 mM pyruvamide, 40 μM ThDP, 80 μM Mg^{+2}, 0.5 Mm NADH, and 5.6 units/mL yeast alcohol dehydrogenase. Inital rates were monitored via the alcohol dehydrogenase/NADH linked assay. The K_m and k_{cat} values were calculated from Lineweaver–Burk plots.

Observation of ThDP-Bound Enamine Intermediate on scpdc1–6. When the scpdc1–6 was incubated with TB, the formation of an enamine with an absorption maximum near 440 nm was observed (Figure 7). The formation and disappearance of the enamine derived from TB on scpdc1–6 and supdc1 were monitored at 440 nm at 5 °C (Figure 8).

DISCUSSION

First, it is important to emphasize that superimposable pH–activity profiles were observed for scpdc1 and supdc1. When purified in parallel, the same purification protocol (Farrenkopf & Jordan, 1992) led to very similar specific activities for the two enzymes from different yeast strains. While we do not have access to the supdc1 sequence (it is a strain used by Anheuser Busch), the sequence of three tryptic fragments of its α "isozyme" indicates greater than 85% identity with the known scpdc1 sequence (Dikdan, unpublished results), and at least 90% of the electron density map of the supdc1 crystal can be satisfactorily fit to the scpdc1 sequence (Dyda, 1992). Furthermore, scpdc1 has also been crystallized recently, and preliminary data indicate that the space group and cell dimensions are essentially the same for the two pdc1 enzymes isolated from different brewers' yeasts.

As can be seen in Table II, at pH 6.2 the specific activity of the fusion enzyme is ca. 30% of the pdc1s, indicating a less efficient catalyst according to the k_{cat}/K_M criterion. The pH optimum shift on scpdc1–6 to more alkaline values is premature to rationalize in the absence of further high-resolution experimental information. The ability to be activated by

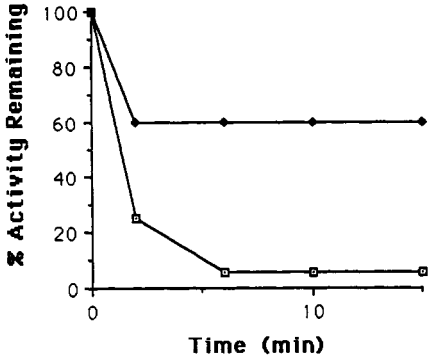


FIGURE 3: Time course of inactivation of supdc1 (□) and scpdc1–6 fusion protein (◆) with MMTS. Thirty units of either enzyme was incubated in 0.5 mL of 2.0 mM MMTS in 0.2 M citrate, pH 6.6, containing 1.0 mM each of ThDP, $MgCl_2$, and EDTA, with 20% ethylene glycol at 25 °C. At the indicated times, aliquots were removed for activity assay with the pH–stat.

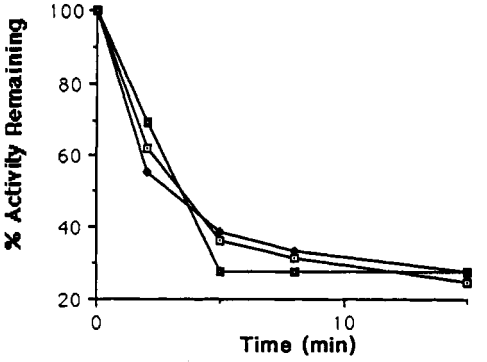


FIGURE 4: Time course of inactivation of brewers' yeast scpdc1 (□), supdc1 (◆), and scpdc1–6 fusion protein (■) with (*p*-nitrobenzoyl)-formic acid. Eighteen units of enzyme was incubated in 1.0 mL of 4.0 mM (*p*-nitrobenzoyl)formic acid in 0.1 M citrate, pH 6.6, containing 1.0 mM each of ThDP, $MgCl_2$, and EDTA, with 20% ethylene glycol at 25 °C. At the indicated times, aliquots were removed for activity assay with the pH–stat.

pyruvamide (as a surrogate for pyruvate, that itself shows substrate activation) is unchanged in the fusion protein, and cooperativity is also evidenced by essentially identical Hill coefficients with respect to pyruvate decarboxylation in scpdc1–6 and supdc1. In view of the distribution of cysteines in scpdc1–6 and scpdc1 (and, presumably, in supdc1), this evidence implicates Cys221 in the activation mechanism, if a Cys side chain is involved at all.

To help interpret the experiments with different inhibitors, the following information is relevant. Dyda (1992) reported that, in the X-ray structure of the α_4 homotetramer of supdc1, all four cysteines are located far from the ThDP coenzyme site in the unactivated form of the enzyme (i.e., in the absence of either substrate or pyruvamide). The doublet cysteines 221 and 222 are closest to ThDP, ca. 20 Å away, on a domain different from those binding ThDP. On the basis of the primary sequence alignments of a large number of ThDP-dependent enzymes, Hawkins et al. (1989) had suggested a putative ThDP binding site (spanning amino acids 440–480 of the scpdc1), very distant from all four cysteines, at least in the primary sequence. The X-ray results have confirmed this hypothesis concerning the location of the ThDP binding motif. In unpublished experiments, Dikdan finds that CPB covalently labels the peptide encompassing Cys221 and Cys222 in supdc1, but not the tryptic peptides encompassing Cys69 and Cys152. Finally, there is no evidence from extensive chemical modification studies reported, especially by Ullrich (1982), that there are any susceptible nucleophiles, other than Cys, whose chemical modification alters the activity of PDC. We conclude from these observations that all of the inactivation observed involves Cys, but that Cys is *not at the catalytic (i.e., ThDP) locus but likely at the regulatory site.* Are the various inhibition studies consistent with this suggestion?

The nonspecific Cys-directed inactivator MMTS is shown to inactivate both supdc1 and scpdc1–6 (Figure 3), but it is much more effective toward supdc1. This suggests that MMTS can probably react with cysteine(s) in addition to Cys 221, but it certainly does react with the latter in scpdc1–6. It is also important to note that scpdc1–6, whose only Cys has been reacted, still retains ca. 60% of its activity, clearly indicating the nonessential nature of Cys221 in catalysis.

Recently, Zeng (1992) exploited benzoylformic acid analogues as alternate substrates for PDC. PDC has a broad substrate specificity and can utilize these compounds well. While (*p*-nitrobenzoyl)formic acid is a good substrate, the product of its reaction, *p*-nitrobenzaldehyde, is a good inhibitor. Although product inhibition for this enzyme is not novel, the K_M for acetaldehyde toward acetoin formation is near 1 M (Chen & Jordan, 1984), and the K_i for *p*-nitrobenzaldehyde is 3.4 mM (Zeng, 1992). At the large concentrations of α-keto acids required in these experiments (due to their high K_M's), *p*-nitrobenzaldehyde turns out to be a good product inhibitor (Zeng, 1992). Figure 4 demonstrates that all three enzymes are nearly equally inhibited by (*p*-nitrobenzoyl)formic acid in a time-dependent manner. Since any Cys modification of the wild-type enzymes significantly reduces the activity, a plausible explanation of this observation is that the Cys221 present in both wild-type and fusion enzymes is the nucleophile that reacts with the electrophile *p*-nitrobenzaldehyde, forming a hemithiolacetal [*p*-$NO_2C_6H_4CH(OH)SCys221$-E]. Such inhibition could be rendered reversible. Accordingly, when alcohol dehydrogenase and NADH were added to a solution of supdc1 that had been ca. 75% inactivated by (*p*-nitrobenzoyl)formic acid, full activity was regained (Zeng, 1992).

During the past decade evidence has been presented from the Rutgers group indicating that the enamine intermediate in Scheme I is indeed on the decarboxylation pathway when derived from the conjugated substrate analogues/inhibitors. The observation at 440 nm of the enzyme-bound intermediate (Kuo & Jordan, 1983; Jordan et al., 1986a; Zeng et al., 1991), its trapping in situ by oxidizing agents (Jordan et al., 1986b; Annan & Jordan, 1990), synthesis of an appropriate analogue with identical λ_{max} (Zeng et al., 1991), and demonstration of its kinetic competence (Jordan et al., 1991; Menon-Rudolph et al., 1992; Zeng, 1992) enable us to interpret the inactivation and spectroscopic data comparing the behaviors of scpdc1–6 and scpdc1 along with supdc1 according to the mechanism drawn in Scheme I. The products of turnover of compounds such as CPB and TB are the corresponding substituted dihydrocinnamic acid and cinnamaldehyde derivatives. Hydrolysis of 2-dihydrocinnamoyl-ThDP is not enzyme catalyzed and may account for weak and temporary inhibition. A transfer of the acyl group from ThDP to ESH is one possible source of long-term inactivation and was suggested by the modification of one of four Cys/subunit in supdc1 concomitant with inactivation (Zeng et al., 1991). While the long-term inactivation may occur by an alternate pathway, the covalent modification of Cys221 and/or Cys222 during inactivation by CPB is also quite clear now from sequencing of a labeled peptide (Dikdan, unpublished results).

When compared with (*p*-nitrobenzoyl)formic acid, CPB and TB have an additional double bond inserted between the keto group and the phenyl ring. CPB and TB show distinctly

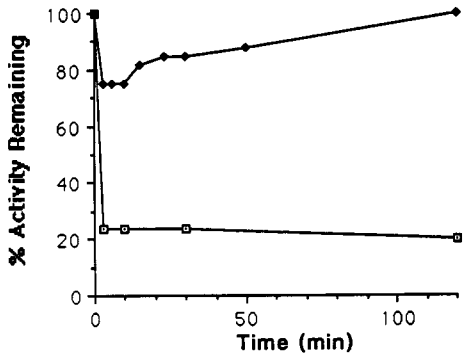


FIGURE 5: Time course of inactivation of supdc1 (□) and of the scpdc1–6 fusion protein (◆) with CPB. Twenty units of either enzyme was incubated in 1.0 mL of 2.0 mM CPB in 0.1 M citrate, pH 6.6, containing 1.0 mM each of ThDP, $MgCl_2$, and EDTA, with 20% ethylene glycol at 25 °C. At the indicated times, aliquots were removed for activity assay with the pH–stat.

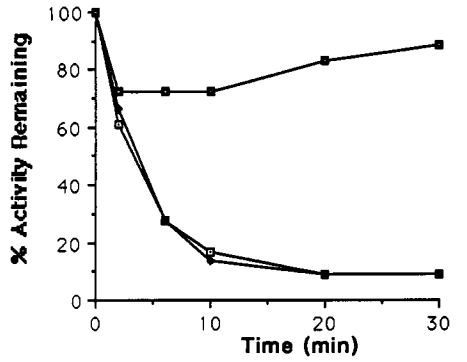


FIGURE 6: Time course of inactivation of brewers' yeast scpdc1 (□), supdc1 (◆), and scpdc1–6 (■) with TB. Eighteen units of scpdc1, supdc1, or scpdc1–6 was incubated in 0.5 mL of 2.0 mM of TB in 0.2 M citrate, pH 6.6, containing 1.0 mM each of ThDP, $MgCl_2$ and EDTA, with 20% ethylene glycol at 25 °C. At the indicated times, aliquots were removed for activity assay with the pH–stat.

different behavior vis-à-vis the two forms of the enzyme and compared to the behavior of (*p*-nitrobenzoyl)formic acid. Unlike supdc1 and scpdc1, which are irreversibly inactivated by CPB (Figure 5) or TB (Figure 6), the scpdc1–6 fusion protein is only weakly and, most importantly, readily reversibly inhibited by both compounds (Figures 5 and 6).

Is there evidence for formation of the enamine intermediate on the decarboxylation pathway in scpdc1–6? Figure 7 demonstrates the type of experiment reported before on supdc1 (Zeng et al. (1991) and references therein), showing that incubation of the scpdc1–6 fusion enzyme with TB enables detection of an enzyme-bound enamine intermediate at 440 nm. Clearly, the enzyme's ability to react with TB according to the mechanistic pathway in Scheme I (operational for both supdc1 and scpdc1) is still retained in scpdc1–6. Why is the scpdc1–6 fusion enzyme inactivated much less than supdc1 by TB and in a readily reversible manner? Figure 8 shows the time course of enamine formation/release that results from mixing TB with supdc1 and scpdc1–6 (reaction with scpdc1 showed behavior identical to that of supdc1 and is not shown). While the enamine is short-lived on the scpdc1–6 fusion enzyme, during the same time span only the buildup of the enamine could be monitored on supdc1. We suggest that the reaction of the scpdc1–6 with TB still follows the pathways shown in Scheme I (i.e., produces both cinnamaldehyde and dihydrocinnamic acid products), and recovery of full activity after decarboxylation (enamine formation) is limited by slow hydrolysis of the dihydrocinnamoyl-ThDP. Simultaneously with turnover, scpdc1 and supdc1 also react

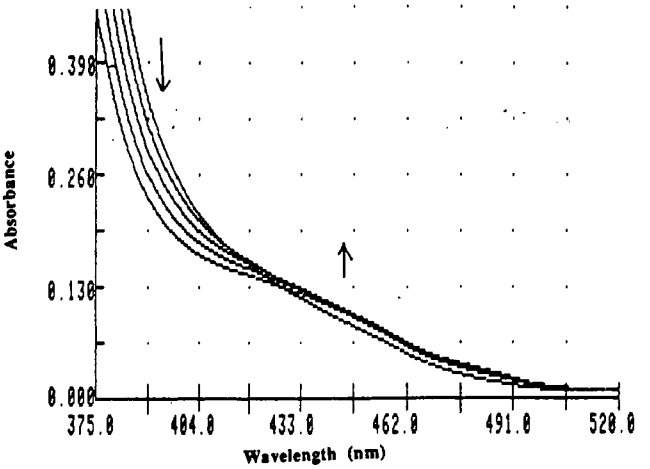


FIGURE 7: Repetitive scan spectrum (Varian DMS 300, 2.0-nm spectra bandwidth, 200 nm/min) between 375 and 520 nm of 0.8 mL of a solution containing 1.0 mM each of ThDP, $MgCl_2$, and EDTA, 1.0 mM TB, and 23 units of scpdc1–6 in 0.1 M citrate, pH 6.6, with 20% ethylene glycol at 5 °C.

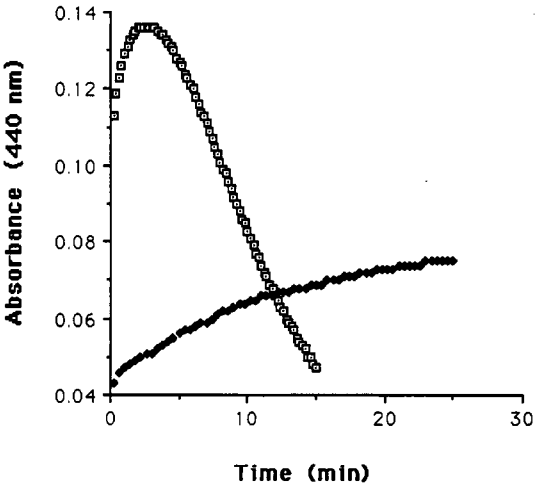


FIGURE 8: Absorbance at 440 nm as a function of time on mixing supdc1 (◆) or scpdc1–6 fusion protein (□) with TB. The reaction contained 1.0 mM TB and 30 units/mL of either enzyme in 0.2 M citrate, pH 6.6, containing 1.0 mM each of ThDP, $MgCl_2$, and EDTA, with 20% ethylene glycol at 5 °C.

with CPB and TB (or their decarboxylated product) at Cys221 and/or Cys222, converting the enzyme to its unactivated form; hence, permanent inactivation results, i.e., the residual activity remaining after inactivation at the regulatory site. It is worth recalling, however, that the cinnamaldehyde products themselves do not inactivate supdc1 (Kuo & Jordan, 1983b). For reasons not clear at present, Cys222 must also be present adjacent to Cys221 for inactivation to result by compounds such as CPB and TB.

In summary, the (*p*-nitrobenzoyl)formic acid experiments, which lead to reversible inhibition by the decarboxylated product *p*-nitrobenzaldehyde, suggest reaction with Cys221 (the only Cys present in the fusion enzyme). Inactivation of pdcs with the longer chain mechanism based inactivators CPB and TB proceeds with Cys221 and/or Cys222 serving as the sites of covalent modification. Only Cys221 is present in the fusion enzyme, and no long-term inactivation results in supdc1–6 from CPB or TB. Our current working hypothesis favors either Cys221 or perhaps the diad formed by Cys221 and Cys222 in a hitherto unrevealed mechanism, constituting the regulatory site of PDC. Modification of one or both of these Cys converts the enzyme from an "activatable" to an "unactivatable" form. The "unactivatable" form, however, is

not totally devoid of activity; rather, the extent of inactivation is roughly proportional to the steric bulk of the reagent used. For example, some of the 2-oxo-4-phenyl-3-butenoic acids studied (e.g., m-CF_3, p-CF_3, and m-NO_2) led to nearly complete inactivation (Annan, 1989), whereas the inhibitors reported here never led to total inactivation.

Perhaps both hypotheses advanced earlier concerning the role of Cys in pyruvate decarboxylase have merit (Schellenberger et al., 1988; Jordan et al., 1988). It is already clear that for the alternate substrates/inhibitors studied by us, at least so far as the fate of the enamine is concerned, the impact of regulation by pyruvamide can be quite dramatic (Jordan et al., 1991; Zeng et al., 1991; Menon-Rudolph et al., 1992; Zeng, 1992). Thus, while activation by pyruvamide may indeed be taking place at a Cys, that Cys is distant from ThDP, and the information is relayed and affects both the rate of formation and release of the enamine intermediate, as well as the regiospecificity of the protonation step (i.e., the ratio of dihydrocinnamic acid to cinnamaldehyde product; Zeng et al. (1991)). The detailed mechanism whereby the enzyme achieves substrate activation will be revealed by a combination of high-resolution data, including the crystal structure.

ACKNOWLEDGMENT

The authors are grateful to the Anheuser Busch Brewing Co. for their continuing generosity in supplying the brewers' yeast for some of the reported studies. S.H. acknowledges receipt of a long-term EMBO fellowship.

REFERENCES

Alvarez, F. J., Ermer, J., Hübner, G., Schellenberger, A., & Schowen, R. L. (1991) *J. Am. Chem. Soc. 111*, 8402–8409.

Annan, N. (1989) Ph.D. Dissertation, Rutgers University Graduate Faculty at Newark, Newark, NJ.

Annan, N., & Jordan, F. (1990) *J. Am. Chem. Soc. 112*, 3222–3223.

Bisswanger, H., & Ullrich, J., Eds. (1991) *Biochemistry and Physiology of Thiamin Diphosphate Enzymes*, pp 1–453, VCH Publishers, New York.

Boiteux, A., & Hess, B. (1970) *FEBS Lett. 9*, 293–296.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Chen, G., & Jordan, F. (1984) *Biochemistry 23*, 3756–3760.

Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B., & Jordan, F. (1990) *J. Biol. Chem. 265*, 17413–17415.

Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B., & Jordan, F. (1991) in *Biochemistry and Physiology of ThDP Enzymes*, pp 115–122, VCH, New York.

Dyda, F. (1992) Ph.D. Dissertation, University of Pittsburgh, Pittsburgh, PA.

Farrenkopf, B., & Jordan, F. (1992) *J. Protein Expression Purif. 3*, 101–107.

Hawkins, C. F., Borges, A., & Perham, R. N. (1989) *FEBS Lett. 255*, 77–82.

Holzer, H., Schultz, G., Villar-Palasi, C., & Juntgen-Sell, J. (1956) *Biochem. Z. 327*, 331–344.

Hohmann, S. (1991a) *J. Bacteriol. 173*, 7963–7969.

Hohmann, S. (1991b) *Curr. Genet. 20*, 373–378.

Hohmann, S. (1991c) in *Biochemistry and Physiology of Thiamin Diphosphate Enzymes* (Bisswanger, H., & Ullrich, J., Eds.) pp 106–114, VCH Publishers, New York.

Hohmann, S., Cederberg, H. (1990) *Eur. J. Biochem. 188*, 615–621.

Hübner, G., Weidhase, R., & Schellenberger, A. (1978) *Eur. J. Biochem. 92*, 175–181.

Jordan, F., Adams, J., Farzami, B., & Kudzin, Z. H. (1986a) *J. Enzyme Inhib. 1*, 139–149.

Jordan, F., Kudzin, Z. H., & Kuo, D. J. (1986b) *Ann. N. Y. Acad. Sci. 471*, 308–309.

Jordan, F., Akinoyosoye, O., Dikdan, G., Kudzin, Z. H., & Kuo, D. J. (1988) in *Thiamine Pyrophosphate Biochemistry* (Schellenberger, A., & Schowen, R. L., Ed.) Vol. 1, pp 79–92, CRC Press, Boca Raton, FL.

Jordan, F., Zeng, X.-P., Menon-Rudolph, S., Annan, N., Barletta, G., Chung, A. C., & Rios, C. B. (1991) in *Biochemistry and Physiology of Thiamin Diphosphate Enzymes* (Bisswanger, H., Ullrich, J., Eds.) pp 34–50, VCH Publishers, New York.

Kluger, R. (1987) *Chem. Rev. 87*, 863–876.

Krampitz, L. O. (1969) *Annu. Rev. Biochem. 38*, 213–240.

Krampitz, L. O. (1970) *Thiamin Diphosphate and Its Catalytic Functions*, Marcel Dekker, New York.

Kuo, D. J., & Jordan, F. (1983a) *J. Biol. Chem. 258*, 13415–13417.

Kuo, D. J., & Jordan, F. (1983b) *Biochemistry 22*, 3735–3740.

Kuo, D. J., Dikdan, G., & Jordan, F. (1986) *J. Biol. Chem. 261*, 3316–3319.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Menon-Rudolph, S., Nishikawa, S., Zeng, X., & Jordan, F. (1992) *J. Am. Chem. Soc. 114*, 10110–10112.

Sable, H. Z., & Gubler, C. J., Eds. (1982) *Ann. N. Y. Acad. Sci. 378*, 7–122.

Schellenberger, A., & Schowen, R. L., Eds. (1988) in *Thiamine Pyrophosphate Biochemistry*, Vols. 1 and 2, CRC Press, Boca Raton, FL.

Schellenberger, A., Hübner, G., & Lehmann, H. (1968) *Angew. Chem., Int. Ed. Engl. 11*, 886–887.

Schellenberger, A., Hübner, G., & Sieber, M. (1988) in *Thiamin Pyrophosphate Biochemistry*, (Schellenberger, A., & Schowen, R. L., Eds.) Vol. 1, pp 113–121, CRC Press, Boca Raton, FL.

Sieber, M., Konig, S., Hubner, G., & Schellenberger, A. (1983) *Biomed. Biochim. Acta 42*, 343–349.

Ullrich, J. (1982) *Ann. N. Y. Acad. Sci. 378*, 287–305.

Weber, K., & Osborne, K. (1969) *J. Biol. Chem. 244*, 4409–4412.

Zeng, X., Chung, A., Haran, M., Jordan, F. (1991) *J. Am. Chem. Soc. 113*, 5842–49.

Zeng, X. (1992) Ph.D. Dissertation, Rutgers University Graduate Faculty, Newark, NJ.