Systematic Study of the Six Cysteines of the E1 Subunit of the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli*: None Is Essential for Activity†

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ABSTRACT: Variants of the *Escherichia coli* 1-lip pyruvate dehydrogenase multienzyme complex (1-lip PDHc) with the C259N and C259S substitutions in the putative thiamin diphosphate-(ThDP-) binding motif of the pyruvate dehydrogenase component (E1, EC 1.2.4.1) were characterized. Single substitutions were made at the five remaining cysteines of the E1 component, creating the C120A, C575A, C610A, C654A, and C770S variants to test the hypothesis that the activity loss that accompanies exposure of the enzyme to fluoropyruvate, bromopyruvate, and 2-oxo-3-butynoic acid is the result of the modification of approximately one cysteine residue per E1 monomer. Surprisingly, all single cysteine E1 variants could be reconstituted with E2−E3 subcomplex and showed PDHc activity ranging from 74% to 96% that of the parental enzyme. The specific activities of C259N and C259S variants of 1-lip PDHc were 58% and 27% relative to that of the parental 1-lip PDHc. All five single cysteine E1 variants, along with the C259N and C259S variants of 1-lip PDHc, could also (1) be inactivated with fluoropyruvate and 2-oxo-3-butynoic acid, (2) were subject to inactivation by the monoclonal antibody 18A9 reported from one of our laboratories, and (3) were subject to regulation by pyruvate and acetyl-CoA. It was therefore concluded that none of the six cysteine residues is essential for the activity of the E1 component or of the complex. When tested with the putative transition-state analogue, thiamin 2-thiothiazolone diphosphate, all but the C259S and C259N variants were very potently inhibited, the stoichiometry for parental E1 being about 1.6 mol of inhibitor/mol of E1 subunit. The C259S and C259N E1 variants required at least 25-fold greater inhibitor concentration to achieve the same level of inhibition. C259 is located in the putative thiamin diphosphate-binding motif of the enzyme [more exactly, it is adjacent to a ligand to the Mg(II) ion]. It is therefore concluded that thiamin 2-thiothiazolone diphosphate is not a transition-state analogue; rather, it is a potent inhibitor of the complex because of a specific interaction with the C259 residue.

The *Escherichia coli* pyruvate dehydrogenase multienzyme complex (PDHc)[1] consists of three different enzymes: pyruvate dehydrogenase (E1, EC 1.2.4.1); dihydrolipoamide transacetylase (E2, EC 2.3.1.12), and lipoamide dehydrogenase (E3, EC 1.8.1.4).

The E1 component catalyzes the thiamin diphosphate-(ThDP-) dependent decarboxylation of pyruvate to 2-α-hydroxyethylidene-ThDP, which in turn undergoes reductive acetyl transfer to form *S*-acetyldihydrolipoamide-E2. ThDP and Mg(II) are essential cofactors that are involved in the regulation of E1 activity (*1*, *2*). The E1 component contains two identical subunits of M_r 99 474 (*3*). There is no X-ray structure for E1, but amino acid sequence alignments suggest that residues 229−260 containing the $GDGX_{26}NCN$ sequence represent the ThDP•Mg binding motif with a β-turn-α-turn-β structure (*4*).

Over many years, evidence suggesting that cysteine residues perform important roles in various ThDP enzymes, including *E. coli* PDHc, has accumulated. Chemical modification studies with the E1 component and E1−E2 subcomplex using organomercurials, such as *p*-mercuribenzoate and *p*-mercuribenzenesulfonate, have revealed that the E1

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[1] Abbreviations: PDHc, pyruvate dehydrogenase multienzyme complex from *Escherichia coli*, consisting of pyruvate dehydrogenase (E1, EC 1.2.4.1); dihydrolipoamide transacetylase (E2, EC 2.3.1.12), and lipoamide dehydrogenase (E3, EC 1.8.1.4); 1-lip and 3-lip PDH complexes refer to pyruvate dehydrogenase complexes with 1- or 3-lipoyl domains in E2; ThDP, thiamin diphosphate; ThTTDP, thiamin 2-thiothiazolone diphosphate; ThTDP, thiamin 2-thiazolone diphosphate; DCPIP, 2,6-dichlorophenolindophenol; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); n_H, Hill coefficient; $[S]_{0.5}$, substrate concentration that yields half the maximal velocity; PMSF, phenylmethanesulfonyl fluoride.

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apoenzyme is completely inactivated when approximately 2 SH groups/dimer have reacted with the mercurials (*5*). The inactivation of *E. coli* PDHc and resolved E1 by fluoropyruvate has further been correlated with the acetyl thioesterification of an SH group/groups on the enzyme (*6*). A similar mechanism was proposed for the inactivation of resolved *E. coli* E1 component by bromopyruvate (*7*). It was recently shown in our laboratories that inactivation of E1 resolved from *E. coli* PDHc by 2-oxo-3-butynoic acid is accompanied by specific modification of 1.3 ± 0.35 SH groups/subunit, probably by the formation of a Michael adduct (*8*).

Pertinent studies with mammalian PDH complexes include (1) chemical modification and kinetics studies on the substrate-dependent inactivation of E1 in the presence of low concentrations of pyruvate, leading to the proposal that 2 SH groups/$\alpha_2\beta_2$ tetramer are important for activity in pigeon breast muscle E1 (*9*) and (2) the labeling of bovine E1 with [^{14}C]-*N*-ethylmaleimide, which revealed that 1 cysteine residue in each α-subunit is alkylated. Sequence analysis further revealed that an E1α peptide containing Cys62 was labeled. This residue is conserved in E1α subunits of PDHc from rat, pig, human, and yeast, all of which have $\alpha_2\beta_2$ structures (*10*).

Cysteine residues have also been extensively studied in other ThDP-dependent 2-oxo-acid decarboxylases. For example, Cys221 is important, probably as a trigger, in the substrate activation pathway of yeast pyruvate decarboxylase (PDC, EC 4.1.1.1; see refs *11*−*14*). The C221S and C221A variants of PDC exhibited hyperbolic Michaelis−Menten behavior, while the wild-type enzyme showed sigmoidal kinetics with a Hill coefficient of about 2.0 (*12*, *14*).

To investigate the functions of the six cysteines in *E. coli* E1 (*3*, *15*), substitutions were made at each residue by site-directed mutagenesis of an E1 expression plasmid to generate C120A, C575A, C610A, C654A, and C770S variants. The C259S and C259N variants were obtained by resolving substituted E1 from the corresponding 1-lip PDH complexes (*16*). The results clearly indicated that none of the six cysteines is "essential" for activity in the classical sense. The variants also helped to further delineate the inhibitory mechanisms of several compounds whose interaction with E1 appears to have been misunderstood.

EXPERIMENTAL PROCEDURES

Materials

The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The Wizard 373 DNA purification system was from Promega and the ABI Prism dye terminator cycle sequencing ready reaction kit with AmpliTag DNA polymerase, FS, was from Perkin-Elmer.

Bacteria and Plasmids. *E. coli* strain TG1 transformed with pGS878 was used for overexpression and site-directed mutagenesis of the *aceE* gene encoding the E1 subunit of *E. coli* PDHc. The E1 expression plasmid (pGS878) was derived from pGS501, which expresses a 1-lip PDHc (E1, 1-lip E2, and E3 subunits) from the IPTG-inducible *tac* promoter (*16*). The 2.57 kb *Sph*I−*Sal*I fragment containing most of the E2 gene and all of the E3 gene was excised from pGS501 and the plasmid was recircularized to generate pGS878 by end-filling the 5′ overhang at the *Sal*I site using DNA polymerase (Klenow fragment), removing the 3′ overhang at the *Sph*I site with T4 DNA polymerase, and blunt-end religation with T4 DNA ligase. In essence, this procedure eliminates the *Sph*I site but recreates the *Sal*I site that is retained downstream of the E1 coding region in pGS878.

The C259S and C259N variants of E1 were isolated from the 1-lip PDH complexes expressed from two derivatives of pGS501, pGS457 and pGS596 (respectively), as described previously (*2*).

Methods

Construction of the C120A, C575A, C610A, C654A, and C770S Variants of E1. Plasmid DNA was purified according to the Wizard 373 DNA purification system from Promega. Mutagenesis reactions were carried out using the QuikChange site-directed mutagenesis kit from Stratagene. The PCR was carried out in the MiniCycler (MJ Research, Inc.) using double-stranded pGS878 DNA, two synthetic mutagenic primers complementary to opposite strands of DNA, and the reagents supplied with the QuikChange site-directed mutagenesis kit for 16 cycles. The following synthetic oligodeoxynucleotides, and their complements, were used as mutagenic primers (mismatched bases underlined and mutant codons in boldface type):

5′-CATTTATGATGTG**GC**<u>**C**</u>TTTAACCAC-TTCTTCCG-3′ (C120A)

5′-GGCGCAGGT**GC**<u>**T**</u>TCCTGGCTGGC-3′ (C575A)

5′-TGGCGATCTG**GC**<u>**C**</u>TGGGCGG-3′ (C610A)

5′-GACTATCCCGAAC**GC**<u>**T**</u>ATCTCTT-ACGACCCG-3′ (C654A)

5′-GATGGTCAGGAT**A**<u>**G**</u>**T**GAACGCTG-GAACATG-3′ (C770S)

The PCR reaction product was treated with *Dpn*I endonuclease to digest the parental DNA template, and plasmids containing the desired mutations were recovered by transforming Epicurian Coli XL1-Blue supercompetent cells and selecting ampicillin-resistant colonies. The presence of mutations was verified by DNA sequencing with the following specific primers, using the ABI Prism dye terminator cycle sequencing ready reaction kit from Perkin-Elmer:

5′-GAGTATCCGGGTAATCTGGAACTGGAAC-3′ (C120A)

5′-CACCCCGCAGGACCGCGAG-3′ (C575A,C610A,C654A)

5′-GTCACGTCCGTGAAGCAGCTGAG-3′ (C770S)

The numbers of each type of mutant recovered were five (C120A), three (C575A), four (C610A), four (C654A), and three of four (C770S).

Overexpression and Purification of Parental E1 and Cysteine Variants of E1. Cultures (20 mL) of *E. coli* strain TG1 transformed with the corresponding plasmids were grown for 16 h at 37 °C in 2× YT medium, containing 100

μg/mL ampicillin, and used to inoculate 1000 mL of the same medium. The cells were grown to $OD_{600} = 0.6-0.7$, induced with IPTG (1 mM), and harvested after a further 6 h and washed with buffer A [20 mM KH_2PO_4 buffer (pH 7.0) containing 0.1 mM PMSF and 1 mM benzamidine hydrochloride] and stored at −20 °C.

All subsequent steps were carried out at 4 °C. Cells were resuspended in buffer B [20 mM KH_2PO_4 buffer (pH 7.0) containing 2 mM ThDP, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 1.0 mM benzamidine hydrochloride, and 1.0 mM EDTA] at 10 mL of buffer/g wet cell weight and sonicated for 3 min (20 s pulsar "on" and 10 s pulsar "off") using the Sonic Dismembrator Model 550 from Fisher Scientific. The extracts were clarified by 30 min of centrifugation at 30000*g*, and ammonium sulfate was added to the supernatant to 0−30% and 30−65% saturation. The 30−65% pellet was dialyzed against buffer C [20 mM KH_2PO_4 (pH 7.0) containing 0.2 mM ThDP, 2 mM $MgCl_2$, 0.1 mM PMSF, and 1.0 mM benzamidine hydrochloride] and applied to a TSK DEAE-5PW HPLC column. The protein was eluted with a linear gradient (0.075−0.5 M) of NaCl in 20 mM KH_2PO_4 buffer (pH 7.5). Fractions containing E1 were combined, concentrated using a Centriprep 30 unit (Amicon), and dialyzed against buffer C.

Parental E1 and E1 variants were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a 12% separating gel according to Laemmli (*17*). Nondenaturing PAGE was performed with 15 mA current at 4 °C using a Bio-Rad Mini Protean II electrophoresis system according to the protocol in bulletin 1822 from Bio-Rad Laboratories. Gels were stained with Coomassie Blue R-250. In a typical experiment, 2−3 μg of E1 was applied to each well of the native gel (4% stacking gel; 16% resolving gel or 4−15% gradient gel from Bio-Rad).

The E1 variants were overexpressed and purified by the same procedures and they behaved exactly like parental E1. All of the variants were stable during 36 days of storage at −20 °C except for the E1 C610A variant. The concentration of that enzyme decreased about 4-fold during that time as a result of precipitation.

The conditions for overproduction of the 1-lip PDH complexes with E1 C259 substitutions were as described elsewhere (*2*, *16*). The C259S and C259N variants of E1 were isolated from the 1-lip PDH complexes on a Sepharose CL-6B column under alkaline conditions as described previously (*2*).

Activity and Related Measurements. The E1 activities of parental E1 and of its cysteine variants were measured according to McNally et al. (*18*), with some modifications (*2*).

The E1 activity was also measured by reconstituting PDHc activity with saturating amount of E2−E3 subcomplex (the ratio of E1 to E2−E3 subcomplex was 1:5) using a Varian DMS 300 spectrophotometer or the Cobas-Bio centrifugal analyzer (Roche Diagnostics, Somerville, NJ), monitoring the pyruvate-dependent reduction of NAD^+ at 340 nm. The reaction medium contained, in 1 mL (DMS 300) or 0.25 mL (Cobas-Bio) test volume, 0.1 M Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 2 mM sodium pyruvate, 2.5 mM NAD^+, 0.1−0.2 mM CoA, 0.2 mM ThDP, and 2.6 mM DTT at 30 °C. The reaction was initiated by adding CoA. Steady-state velocities were taken from the linear portion of the progress curve. One unit of activity is defined as the amount of NADH produced (micromolar per minute per milligram of E1).

The Michaelis−Menten constant K_m, $S_{0.5}$, and Hill coefficient (n_H) were obtained by fitting the steady-state rates at different concentrations of pyruvate to the Hill equation using the Delta Graph (Pro4) computer program. The steady-state rates were also analyzed using the Eadie−Scatchard and Lineweaver−Burk plots.

Inactivation of Parental E1 and Variants by Fluoropyruvate. Parental E1 and its variants (0.267 μM) were incubated with 1 mM ThDP and 5 mM $MgCl_2$ and 0.1 mM fluoropyruvate in 50 mM KH_2PO_4 buffer, pH 7.5, at 25 °C in a total volume of 0.5 mL. The initial activity (100%) was assayed prior to the addition of fluoropyruvate. Samples (50 μL) were withdrawn periodically and diluted into 1 mL of assay solution, containing E2−E3 subcomplex and all components necessary for assaying the PDHc activity.

1-Lip PDHc (0.049 mg), or its C259N (0.049 mg) or C259S variant (0.092 mg) was incubated in 20 mM KH_2PO_4, pH 7.5, with 1 mM ThDP, 5 mM $MgCl_2$, and 0.001−0.1 mM concentrations of fluoropyruvate at 25 °C in a total volume of 0.25 mL. After 30 min of incubation, 20 μL aliquots were withdrawn and the PDHc activity was assayed using the Cobas-Bio instrument.

Inactivation of Parental E1 and Variants by 2-Oxo-3-butynoic Acid. Conditions for inactivation of parental E1 and E1 variants by 2-oxo-3-butynoic acid were the same as described previously (*8*).

Inactivation of Parental E1 and Its Cysteine Variants by Thiamin 2-Thiothiazolone Diphosphate. ThTTDP was dissolved in 20 mM KH_2PO_4 buffer, pH 7.0, and its concentration was determined at 319 nm using the $\epsilon_{319} = 10\,900\ M^{-1}\ cm^{-1}$ (*19*). For inactivation, the parental E1 or its variants (0.528 μM) were incubated in 50 mM KH_2PO_4 buffer (pH 7.0) with 2 mM $MgCl_2$ in a total volume of 0.5 mL at 25 °C. The initial activity was measured prior to the addition of 1 μM ThTTDP (100%). At different times, aliquots (50 μL) were withdrawn and diluted into 1 mL of assay medium, containing the E2−E3 fraction (molar ratio E1:E2−E3 of 1:5) and all components required for assaying the PDHc reaction. The reaction was initiated by addition of 0.1 mM CoA.

For titrating the active sites of parental E1 with ThTTDP, E1 (1.09 μM) was incubated in 50 mM KH_2PO_4 (pH 7.0) with concentrations of ThTTDP in the range of 0.1−50 μM and 2 mM $MgCl_2$ in a total volume of 0.1 mL at 25 °C. After 40 min, 10 μL aliquots were withdrawn and diluted into 0.25 mL of assay solution, containing E2−E3 subcomplex and all components required for assaying the PDHc activity. Titration of the C259N E1 variant (0.885 μM) resolved from the 1-lip PDHc with ThTTDP was carried out under the same conditions.

Synthesis of Thiamin 2-Thiothiazolone Diphosphate. Orthophosphoric acid (85%, 5 mL in a 50 mL round-bottom flask) was polymerized by heating until the solution became faintly cloudy (*20*, *21*). Upon cooling to room temperature, a clear syrupy liquid resulted, to which was added thiamin 2-thiothiazolone (1.0 g). This mixture was then heated at 100 °C for 15 min while being stirred with a glass rod and then cooled to room temperature; then a mixture of 4 mL of water and 1 mL of phosphoric acid was added and the mixture was stirred for 2 h. Next, the pH was adjusted to

Table 1: Activities and Kinetic Parameters for Pyruvate Binding by Parental E1, Variants of E1 with Single Cysteine Substitutions, 1-Lip PDHc, and the C259S and C259N Variants of 1-Lip PDHc[a]

enzyme	substitution	pyruvate:NAD^+ oxidoreductase activity (units/mg of E1)[b]	relative activity (%)	E1 activity[c] (milliunits/mg)	K_m[d] (mM)		n_H[e]	
					−	+	−	+
E1		7.0 ± 0.15	100	20.5 ± 2.7	0.21	0.16	0.96	1.14
E1	C120A	6.7 ± 0.31	96	22.5 ± 0.9	0.15	0.17	1.05	1.22
	C575A	6.8 ± 0.28	97	25.4 ± 2.0	0.14	0.10	0.98	1.32
	C610A	6.4 ± 0.54	91	18.9 ± 0.9	0.09	0.08	1.18	1.41
	C654A	5.7 ± 0.25	81	27.9 ± 3.6	0.12	0.15	1.06	1.23
	C770S	5.2 ± 0.15	74	21.5 ± 1.5	0.15	0.16	1.07	1.18
1-lipPDHc		6.8 ± 0.37	100		0.27	0.73	1.10	1.40
	C259S	1.85 ± 0.29	27		1.10	3.25	1.07	1.61
	C259N	3.94 ± 0.23	58		0.28	1.08	1.26	1.61

[a] Activities are averages from five repeat measurements for each preparation. [b] Activities of 1-lip PDHc and the C259S and C259N variants of 1-lip PDHc were defined as units per milligram of total protein. [c] E1 activity was assayed according to E1-specific assay as described in Experimental Procedures. [d] Apparent K_m. Left column (−), no acetyl-CoA; column (+), right 0.65 mM acetyl-CoA. [e] Hill coefficient. Left column (−), no acetyl-CoA; right column (+), 0.65 mM acetyl-CoA.

2.0 with 6 N NaOH and the solution was applied to a column of Amberlite CG-50, H^+ cation-exchange resin and eluted with water, the fractions being monitored by TLC on cellulose plates. A mixture (10:1:6) of EtOH:*n*-BuOH:0.5 M sodium citrate (pH 4) was used to develop the plates. Three spots were detected on TLC, with R_f values of 0.76, 0.68, and 0.41 for thiamin 2-thiothiazolone, thiamin 2-thiothiazolone monophosphate, and thiamin 2-thiothiazolone diphosphate, respectively. ^{1}H NMR (D_2O) δ 2.26 (3 H, s), 2.50 (3 H, s), 3.15 (2 H, t), 4.20 (2 H, t), 5.40 (2 H, s), 7.58 (1 H, s). ^{31}P NMR (D_2O/vs phosphoric acid) δ −3.20 (α), −12.9 (β).

RESULTS

Characterization of Parental E1 and Its Cysteine Variants. Parental E1 and variant E1 proteins were overexpressed from the IPTG-inducible expression plasmid pGS878 and its derivatives. Typical yields of purified protein were 14 (parental E1), 13 (C120A), and 5−7 (C575A, C610A, C654A, and C770S) mg/L of induced culture. Addition of ThDP to the buffers used for purification was essential for enzyme activity. With parental E1, purification in the absence of ThDP produced no active enzyme, nor was activity observed even after prolonged incubation with saturating concentrations of ThDP (1 mM) in the presence of $MgCl_2$ (5 mM). Apparently, ThDP is essential for correct folding of this enzyme.

The parental E1 overexpressed from expression plasmid pGS878 was correctly refolded according to the following criteria:

(1) The parental E1 is a dimer. A single peak was obtained from a TSK G3000-SW size-exclusion column with a retention time of 35.86 min, compared with 35.57 min for E1 resolved from 3-lip PDHc. The apparent molecular masses (187 and 199 kDa) deduced for these proteins indicate that they are dimeric, in accord with earlier reports (*22*, *23*).

(2) The parental E1 reconstituted with saturating amount of E2−E3 subcomplex resolved from 1-lip and 3-lip PDH complexes gave PDHc activity similar to that obtained with E1 component resolved from these complexes.

(3) The parental E1 and its variants were recognized by monoclonal antibody 18A9 (elicited to PDHc and shown to interact with E1; refs *18* and *24*) and 99.7−100% inhibited (data not shown), as observed with 1-lip and 3-lip PDH complexes (98% and 99%, respectively).

(4) The parental E1 and its variants exhibited electrophoretic behavior similar to that of E1 resolved from 1-lip PDHc in PAGE under nondenaturing conditions (data not presented).

(5) The parental E1 exhibited kinetics similar to those of E1 resolved from the 1-lip PDHc (presented below).

Site-Directed Mutagenesis. Kinetic Properties of Single Cysteine Variants of E1. The amino acid analysis (*15*) and deduced amino acid sequence (*3*) indicate that there are 6 cysteine residues/subunit of E1. Site-directed mutagenesis has been used to determine which, if any, are essential for E1 activity. Variants of the 1-lip PDHc with C259N and C259S substitutions in the putative ThDP-binding motif of the E1 subunit were first characterized (*2*, *16*). The specific activities were 27% (C259S) and 58% (C259N) relative to that of the parental 1-lip PDHc. It was shown that the C259N variant could be saturated by ThDP but the K_m for ThDP was about 13-fold higher, and the enzyme still exhibited an activation lag phase, like the parental 1-lip PDHc. The C259S variant could not be saturated by ThDP, nor did it exhibit an activation lag phase, but it resembled the parental enzyme with respect to pyruvate binding (*2*). Here, the five remaining cysteine residues were replaced by alanine and/or serine using an E1 expression plasmid to generate the following singly substituted variants: C120A, C575A, C610A, C654A, and C770S .

The activities and kinetic parameters for pyruvate binding by parental E1 and the variants of E1 are compared in Table 1. All of the E1 variants could be successfully reconstituted with E2−E3 subcomplex and the corresponding PDHc activities were not significantly different from that observed with parental E1. The E1 activities measured with the E1-specific assay (*18*) gave similar values for all variants. The $S_{0.5}$ and n_H values for the E1 variants for pyruvate binding (Table 1) suggest that replacing single cysteine residues has little effect on enzyme activity and pyruvate binding. Compared with previous findings with E1 resolved from the PDHc (*2*) where K_m = 0.11 mM and n_H = 1.0, it would appear that parental E1 and the E1 variants have essentially the same kinetic properties with respect to pyruvate. ThDP binding was not investigated because the parental E1 and

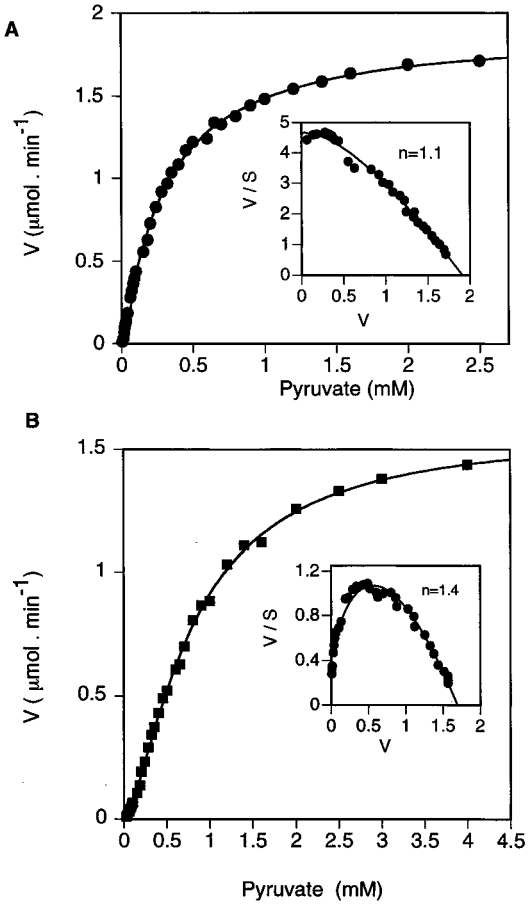

FIGURE 1: (A) Michaelis−Menten plots for 1-lip PDHc. The reaction mixture (0.25 mL) in 50 mM Tris-HCl buffer (pH 8.0) contained 2.5 mM NAD^+, 2.6 mM DTT, 0.2 mM ThDP, 1 mM Mg(II), and 0.13 mM CoA. The reaction was initiated by addition of enzyme (1.6 μg). Inset: Eadie−Scatchard plot. (B) Michaelis−Menten plot for 1-lip PDHc in the presence of 0.65 mM acetyl-CoA. Reaction conditions were the same as in panel A. Inset: Eadie−Scatchard plot.

its variants were purified in the presence of a saturating concentration of ThDP.

Feedback Inhibition of PDHc and Variants by Acetyl-CoA. It had been shown that acetyl-CoA, the E2 product, inhibits the PDHc activity competitively with pyruvate via the E1 component (*25*, *26*). Noncompetitive inhibition with respect to pyruvate was obtained using the E1 assay (*27*). Also, during studies on the effect of pyruvate and acetyl-CoA on PDHc activity, it was noted that 0.47 mM acetyl-CoA increased the n_H for pyruvate from 1.85 to 2.8 (*26*). Studies using electron spin resonance spectroscopy with a spin-labeled acetyl-CoA analogue revealed 2 binding sites/E1 dimer, distinct from the pyruvate and ThDP binding sites and overlapping the acetyl-CoA binding sites (*27*).

In our hands, the 3-lip and 1-lip PDH complexes exhibited weak positive cooperativity with pyruvate; $S_{0.5} = 0.21$ mM ($n_H = 1.1$) and 0.22 mM ($n = 1.24$), respectively (*2*). The data for 1-lip PDHc (Figure 1A) and 3-lip PDHc (Figure 2A) are not very different from the values of $K_m = 0.11$ mM ($n_H = 1.0$) obtained for resolved E1 (*2*) and the value of $S_{0.5} = 0.21$ mM ($n_H = 0.96$) obtained for parental E1 (Table 1). It was shown that acetyl-CoA at 0.13 and 0.39 mM concentrations increased the n_H for 3-lip PDHc to 1.37 and 1.60, respectively, and $S_{0.5}$ for pyruvate to 0.42 and 0.27 mM, respectively (in the absence of acetyl-CoA the $S_{0.5}$ for pyruvate was 0.11 mM) (Figure 2). For the 1-lip PDHc, n_H was 1.4 and $S_{0.5}$ was 0.73 mM in the presence of 0.65 mM CoA, compared with $n_H = 1.1$ and $S_{0.5} = 0.27$ mM in the absence of acetyl-CoA (Figure 1). Similar observations were made with the C259N and C259S variants (data not presented): in the presence of 0.65 mM acetyl-CoA the n_H increased from 1.26 to 1.61 for the C259N variant while its $S_{0.5}$ increased from 0.28 to 1.08 mM; and n_H increased from 1.07 to 1.61 for the C259S variant while its $S_{0.5}$ value increased from 1.1 to 3.25 mM, indicating that Cys259 is not critical for the regulation of E1 activity by acetyl-CoA.

As can be seen in Table 1 for parental E1 and its cysteine variants, the presence of 0.65 mM acetyl-CoA does not influence significantly the values of $S_{0.5}$ for pyruvate (obtained by fitting the steady-state rates at different concentrations of pyruvate as described in Methods) but it does increase the values of n_H slightly, indicating weak positive cooperativity. An analysis of the variation in reaction rates with pyruvate concentration in the presence of 0.65 mM acetyl-CoA using the Eadie−Scatchard and Lineweaver−Burk plots (not presented) revealed positive cooperativity. Data are presented for the C575A variant of E1 in Figure 3. Similar results were obtained for the C120A, C610A, C654A, and C770S variants of E1 (data not presented). The allosteric inhibition by acetyl-CoA was not as pronounced when PDHc was reconstituted from E1 and E2−E3 subcomplex, as with preformed 1-lip and 3-lip PDH complexes and the E1 C259N and C259S variants of 1-lip PDHc (compare data for parental E1 and E1 variants with data for 1-lip PDHc and variants in Table 1) .

The experimental data suggest that none of the cysteine residues of E1 is essential for pyruvate binding or for regulation of enzyme activity by acetyl-CoA. They also show that allosteric inhibition by acetyl-CoA is less pronounced with reconstituted PDHc than with preformed complexes.

Inhibition of E1 Variants by Fluoropyruvate. It had been reported that inactivation of the *E. coli* E1 component by fluoropyruvate (*6*), bromopyruvate (*7*) and 2-oxo-3-butynoic acid (*8*) is accompanied by specific modifications of 1.2−1.37 SH groups/subunit. A careful analysis of the inhibition of PDHc and resolved E1 by fluoropyruvate revealed that a thiol ester bond was being formed. When ^{14}C-fluoropyruvate was used to inactivate the PDHc, subsequent incubation with 1 M NH_2OH at pH 8.0 removed 65% of the label after 60 min and 73% after 120 min. The inhibition product lost the label at pH 12.6, but the label was retained at pH 2.5. Dihydrolipoamide protected PDHc from inactivation by fluoropyruvate. These results led Flournoy and Frey (*6*) to suggest that a sulfhydryl group was being acetylated during inactivation. On the basis of this report, fluoropyruvate was tested on the cysteine variants in an attempt to identify the residue being modified.

It was found that the 1-lip PDHc and its C259S and C259N variants were inhibited by 0.001−0.1 mM fluoropyruvate (residual activities after 30 min of incubation in the presence of 0.1 mM fluoropyruvate were 17%, 14%, and 11%, respectively; data not shown). The remaining five variants with single cysteine substitutions and parental E1 were likewise inhibited (panels A and B of Figure 4 present

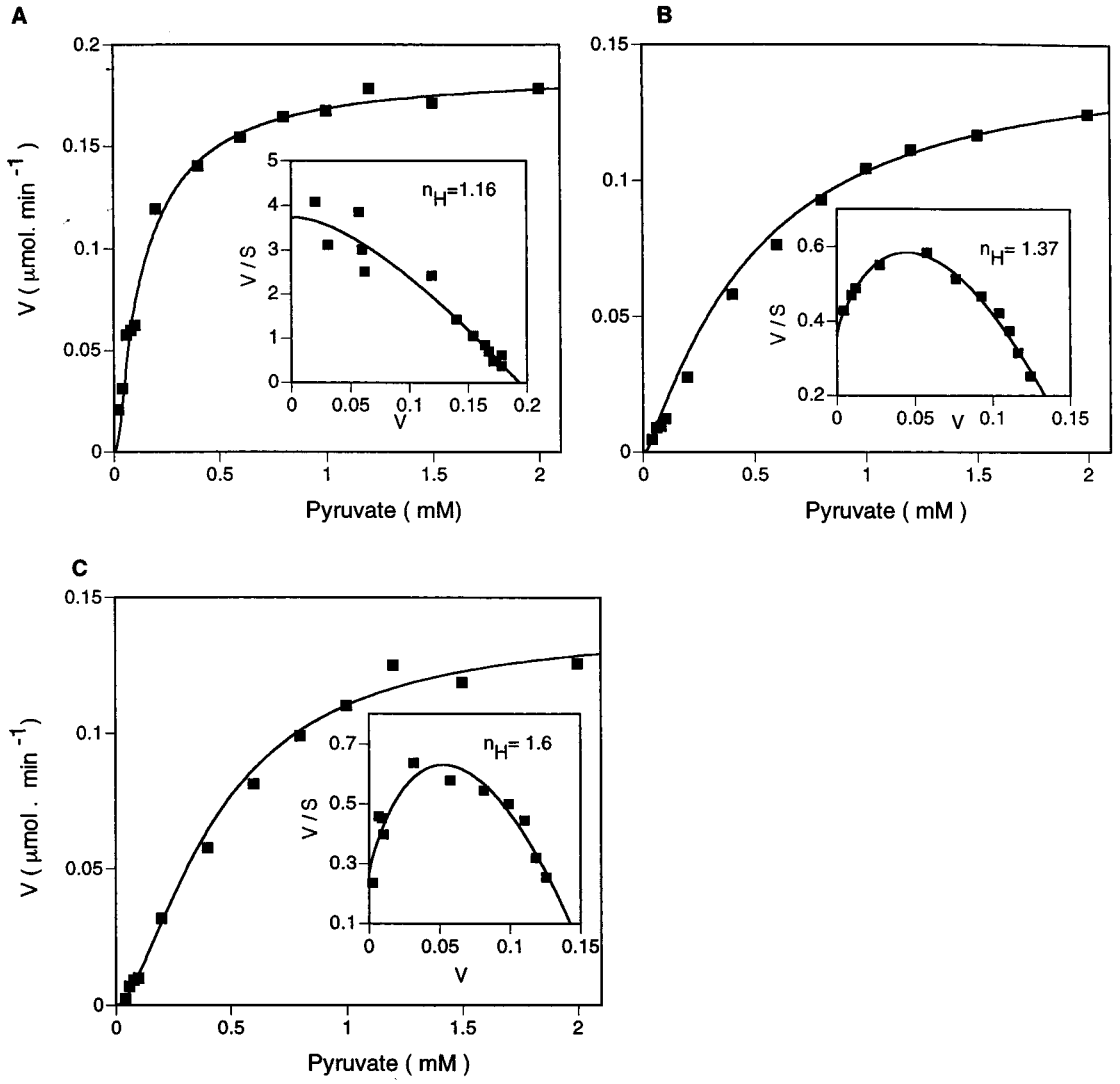

FIGURE 2: (A) Michaelis−Menten plot for 3-lip PDHc. Reaction conditions were the same as in Figure 1A. The reaction was initiated by addition of enzyme (1 μg). Inset: Eadie−Scatchard plot. (B) Michaelis−Menten plot for 3-lip PDHc in the presence of 0.13 mM acetyl-CoA. Inset: Eadie−Scatchard plot. (C) Michaelis−Menten plot for 3-lip PDHc in the presence of 0.39 mM acetyl-CoA. Inset: Eadie−Scatchard plot.

representative data for the parental E1 and its C575A variant). The activities of parental E1 and its variants were stable during 40 min of incubation in the absence of fluoropyruvate, thereby excluding any nonspecific inactivation. The kinetics of inactivation exhibited biphasic behavior: a rapid initial phase being followed by a slower phase. The first-order k_{app} of inactivation values were calculated for the slower phase (Table 2), since the fast initial phase of inactivation was complete within the first minute of incubation.

According to Flournoy and Frey (*6*), the complex kinetics of inactivation by fluoropyruvate of *E. coli* PDHc and of E1 resolved from this complex may be due to depletion of inhibitor concentration concomitant with the inactivation. It was shown that, at high concentrations of fluoropyruvate, the initial phase of inactivation was followed by a plateau level, and upon addition of another aliquot of inhibitor a second lower plateau was reached. At high concentrations of inhibitor, intervention of the "carboligase" side reaction at E1 was proposed, which would lead to the formation of fluoroacetofluorolactate, the product of benzoin condensation between the 2-fluoro-1-hydroxyethylidene-ThDP and a second molecule of fluoropyruvate (*6*), thereby decreasing the concentration of fluoropyruvate. It was shown in our laboratories (data not shown) that, at 0.001−0.1 mM concentrations of fluoropyruvate, the kinetics of inactivation of 3-lip PDHc and of the C259N, C259S, and N258Q E1 variants of 1-lip PDHc were complex and reached a plateau. Similar plateaus were observed for the inhibition of E1 resolved from *E. coli* PDHc by 0.015-0.062 mM fluoropyruvate, suggesting that the concentration of fluoropyruvate was depleted during the incubation with enzyme. Therefore, the apparent "biphasic" kinetics of inactivation of parental E1 and variants of E1 by fluoropyruvate more likely reflects a slow and complex transformation of the enzyme•fluoropyruvate complex to inactive E*•I complex, rather than chemical modification of two cysteine residues with different affinities for fluoropyruvate, as we also proposed for inhibition of the PDHc and E1 resolved from PDHc by 2-oxo-3-butynoic acid (*8*).

Also, if two cysteine residues, acting in concert, were responsible for inactivation by fluoropyruvate, and one of the two was substituted by a different amino acid, then in at least two of the six cases the biphasic kinetics of inactivation should change to a simple monophasic one during the time

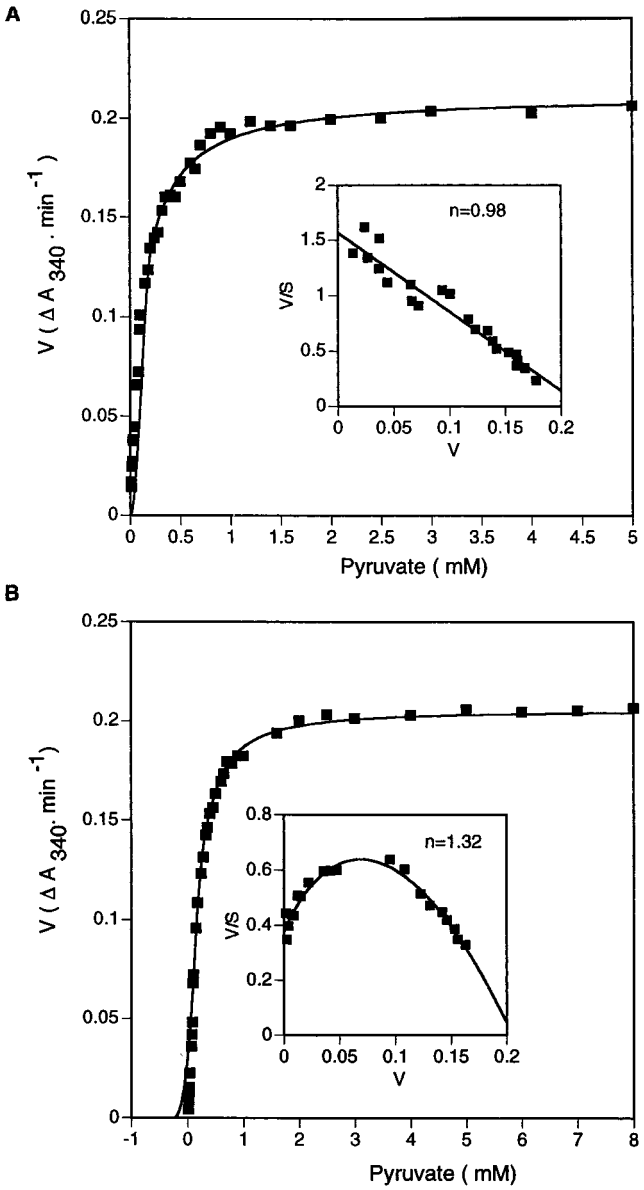

FIGURE 3: (A) Michaelis−Menten plot for the C575A-E1 variant. The reaction mixture (0.25 mL) in 0.1 M Tris-HCl (pH 8.0) contained 2.5 mM NAD^+, 2.6 mM DTT, 0.2 mM ThDP, 1 mM Mg(II), and 0.13 mM CoA. The reaction was initiated by addition of a mixture containing C575A-E1 (0.7 μg) and E2−E3 subcomplex (4 μg). Inset: Eadie−Scatchard plot. (B) Michaelis−Menten plot for the C575A variant of E1 in the presence of 0.65 mM acetyl-CoA. Inset: Eadie−Scatchard plot.

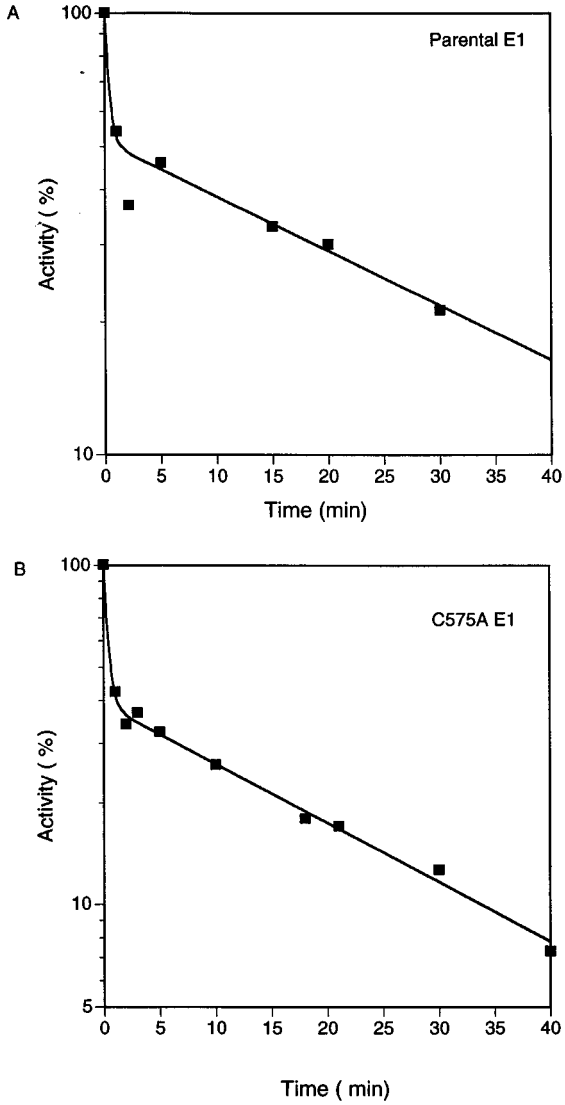

FIGURE 4: Inactivation of parental E1 (A) and of its C575A variant (B) by fluoropyruvate. Parental E1 or its C575A variant (0.267 μM) were incubated in 50 mM KH_2PO_4 buffer (pH 7.5) containing 1 mM ThDP, 5 mM $MgCl_2$, and 0.1 mM fluoropyruvate at 25 °C in a total volume of 0.5 mL. The initial activity (100%) was assayed prior to the addition of fluoropyruvate. Samples (50 μL) were withdrawn periodically and diluted into 1 mL of reaction medium containing E2−E3 subcomplex (the ratio E1:E2−E3 was 1:5) and all components required for assay of PDH complex activity (see Methods). The reaction was initiated by the addition of CoA (0.1 mM).

of incubation. However, all of our variants exhibited similar kinetic patterns of inactivation.

Overall, the results indicate that none of the six cysteine residues in the E1 subunit is required for inactivation by fluoropyruvate and that the mechanism of inactivation is more complex than reported (*6*).

Inhibition of E1 Variants by 2-Oxo-3-butynoic Acid. In a manner similar to that used for the parental E1, the C120A, C259N, C575A, C654A, and C770S variants of E1 were treated with 2-oxo-3-butynoic acid. The C610A variant of E1 was not tested with 2-oxo-3-butynoic acid because of its small activity and instability, as mentioned in Methods. It was shown that the activity of parental E1 and of E1 variants decreased after 20 min of incubation at 25 °C with 2-oxo-3-butynoic acid in a concentration-dependent manner (see Figure 5). In the presence of 0.5 mM 2-oxo-3-butynoic acid the residual activities were 2.5%, parental E1; 4.9%, C120A; 11%, C259N; 41%, C575A; 13%, C654A; and 42% C770S. The activity of the C575A and C770S variants did not change

Table 2: Kinetic Data for Inhibition of Parental E1 and Its Variants by Fluoropyruvate (0.1 mM) and ThTTDP (1 μM)[a]

enzyme	substitution	fluoropyruvate k_{app} (min^{-1})	ThTTDP k_{app} (min^{-1})
E1		0.029	0.009
E1	C120A	0.030	0.039
	C575A	0.040	0.030
	C610A	0.045	0.008
	C654A	0.032	0.081
	C770S	0.034	0.021

[a] See Experimental Procedures for conditions.

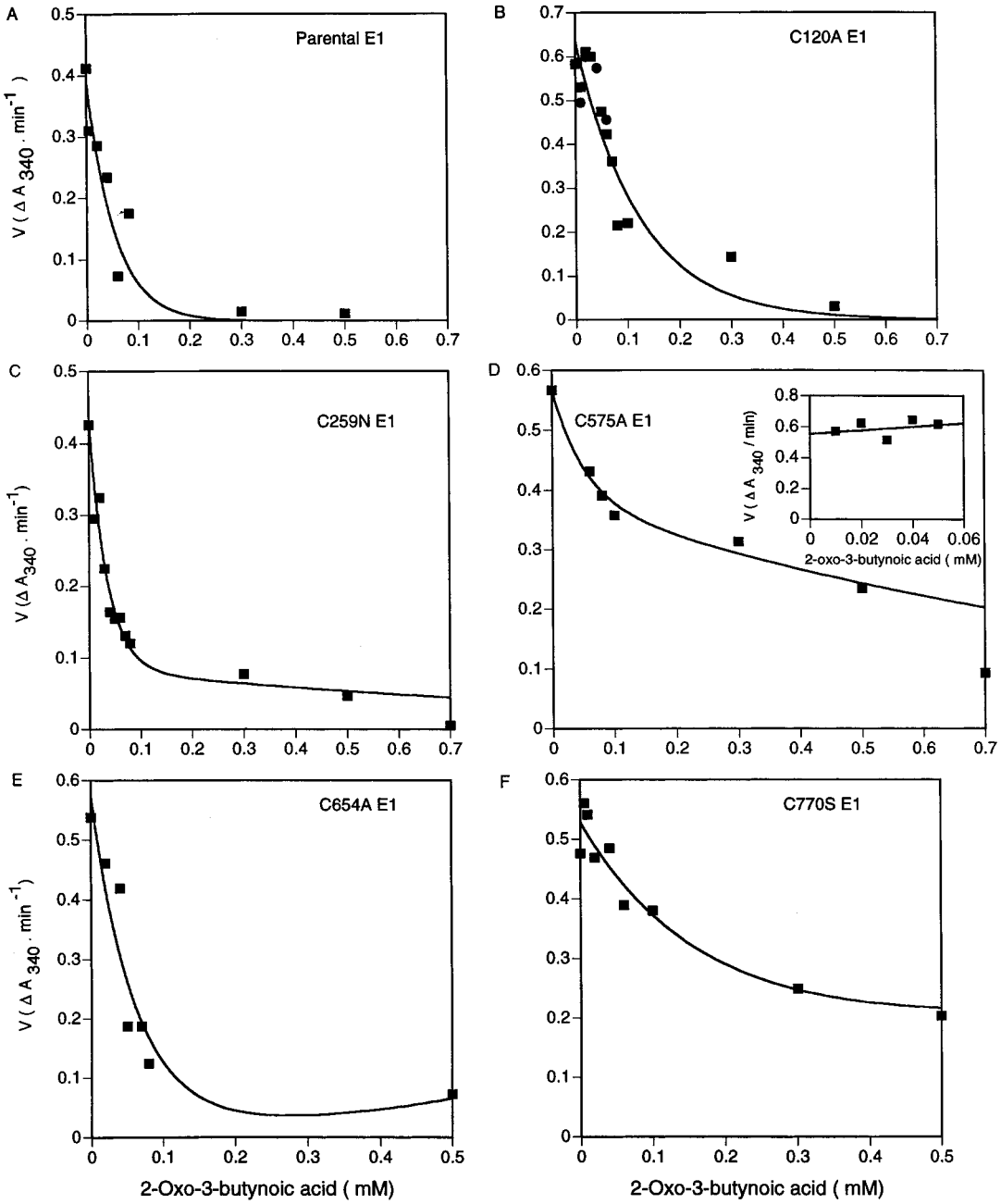

FIGURE 5: Inactivation of parental E1 and its cysteine variants by 2-oxo-3-butynoic acid. Parental E1 (A) and its C120A (B), C575A (D), C654A (E), and C770S (F) variants (0.38 μM) and resolved C259N E1 (C, 0.89 μM) were incubated in the dark with 5 mM $MgCl_2$, 1 mM ThDP, and different concentrations of 2-oxo-3-butynoic acid in 20 mM KH_2PO_4 buffer (pH 7.5) at 25 °C in a total volume of 0.1 mL. Samples (10 μL) were withdrawn after 20 min of incubation and diluted into 0.25 mL of assay solution containing E2−E3 subcomplex and all components required to assay the PDH complex activity. The reaction was initiated by the addition of CoA (0.13 mM).

significantly in the presence of 0.01−0.05 mM concentrations of 2-oxo-3-butynoic acid as compared with parental E1 (see Figure 5 for representative data).

Next, experiments were carried out to determine the number of SH groups modified by 2-oxo-3-butynoic acid in the C259N and C575A variants. Previously, for resolved E1 it was shown that under denaturing but nonreducing conditions (8 M urea) 5.13 SH groups/E1 monomer were titrated by DTNB (*8*). Inactivation of E1 by 2-oxo-3-butynoic acid was accompanied by specific modification of 1.3 SH groups/E1 monomer (*8*). Prior to reaction with 2-oxo-3-butynoic acid, the C259N and C575A variants had 4.4 ± 0.34 and 4.2 ± 0.27 SH groups/E1 monomer. After reaction with 2 mM 2-oxo-3-butynoic acid, the residual activities were 2.8% for C259N and 6.4% for the C575A variant. After inactivation with 2 mM 2-oxo-3-butynoic acid, 2.3 ± 0.01 SH groups/C259N monomer and 3.8 ± 0.29 SH groups/C575A monomer were found, indicating that inactivation of the C259N variant is accompanied by modification of 2.1 SH groups/monomer (4.4 − 2.3), and of the C575A variant by modification of 0.42 SH groups/monomer (4.2 − 3.8). On the basis of the activity remaining after inhibition (A_t = 41% for the C575A variant) and the number of SH groups modified during the inactivation, we cannot exclude the possibility that C575 and/or C770 (A_t = 42%, cysteine residues were not titrated by DTNB) is involved in the mechanism of inactivation by 2-oxo-3-butynoic acid. It is likely that the reaction of the inhibitor that leads to

inactivation is at a site different from C575 and C770, although these side chains also react with the inhibitor.

Inhibition of E1 Variants by the Putative Transition-State Analogue ThTTDP. It was shown by Gutowsky and Lienhard (*19*) and Williams et al. (*28*) that PDHc from *E. coli* is potently inhibited by the ThDP analogue, thiamin 2-thiazolone diphosphate (ThTDP, where the C2═O bond replaces the key C2H) and thiamin 2-thiothiazolone diphosphate (ThTTDP, where the C2═S bond replaces C2H). ThDP protected the enzyme from inactivation by ThTDP, suggesting it was bound at the ThDP binding site. The K_d for ThTDP was 5×10^{-10} M, compared to $K_d = 1 \times 10^{-5}$ M for ThDP, suggesting that the coenzyme analogue binds much more tightly than ThDP and prompting Gutowsky and Lienhard to designate these two compounds as "transition-state analogues" for the PDHc. The analogy is presumably related to the presence of a C═C bond in the 2-α-hydroxyethylidene-ThDP intermediate. Gutowsky and Lienhard (*19*) mentioned that ThTTDP and ThTDP gave similar results and that both compounds may be useful for quantitative titration of the coenzyme binding sites. It was important, therefore, to test whether the single cysteine variants are still subject to potent inactivation by such compounds.

Although the parental E1 and its variants were purified in the presence of saturating concentrations of ThDP, a comparison of the holoenzyme activities of the reconstituted PDHc in the presence and absence of ThDP could be made: 20% in parental E1; 26% in C120A; and about 50% in the C575A, C610A, C654A, and C770S variants, indicating that each contains different amounts of tightly bound ThDP. The time-dependent inhibition of parental E1 and its variants by 1 μM ThTTDP exhibited biphasic behavior (see Figure 6, panels A and B, for parental E1 and its C770S variant), similar to those observed with fluoropyruvate. The k_{app} values calculated for the slow phase of inactivation are listed in Table 2; they indicate that ThDP has no substantial influence on the kinetics of inactivation by ThTTDP, nor does replacement of cysteine by alanine or serine (C770S) at these five positions affect significantly the binding of ThTTDP to the coenzyme binding sites.

A titration of coenzyme binding sites of parental E1 by ThTTDP is presented in Figure 7. Three different preparations of E1 containing different amounts of tightly bound ThDP were used; however, only that with 2.99% holoenzyme activity was useful. Binding about 1.6 mol of ThTTDP/mol of E1 monomer was sufficient to inactivate the enzyme (Figure 7), indicating that there are at least 2 coenzyme binding sites/E1 dimer. The other samples of E1 required even more ThTTDP to displace ThDP from the active sites and hence were not useful.

With E1 resolved from the C259N E1 variant of 1-lip PDHc, concentrations of ThTTDP in the range 0.2−3 μM (Figure 8, inset) or even at 5 μM (data not presented) had no effect on enzyme activity. The titration curve (Figure 8) shows that about 25 mol of ThTTDP/mol of C259N E1 is required to inactivate the enzyme, compared to 3.2 mol/mol of parental E1. The C259S variant of 1-lip PDHc likewise resisted inhibition by 0.2−10 μM ThTTDP (Figure 9). We conclude that Cys259, located in the ThDP binding fold, is important for ThTTDP binding.

To understand the mechanism of inactivation by ThTTDP, three different preparations of parental E1 were incubated

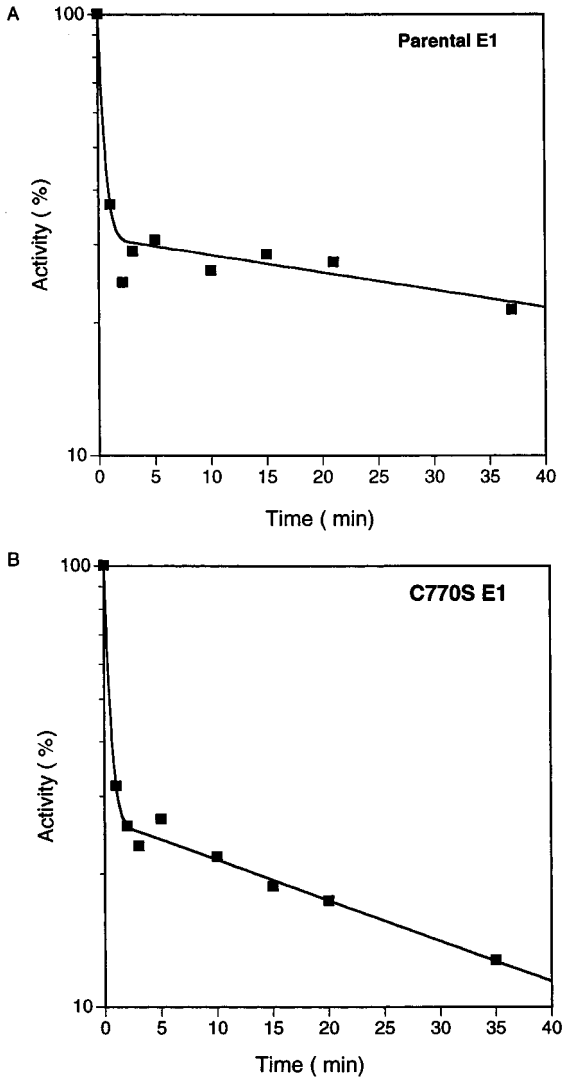

FIGURE 6: Inactivation of parental E1 (A) and its C770S variant (B) by ThTTDP. The parental E1 or its C770S variant (0.528 μM) was incubated in 50 mM KH_2PO_4 buffer (pH 7.0) with 2 mM $MgCl_2$ and 1 μM ThTTDP at 25 °C in a total volume of 0.5 mL. Periodically, 50 μL aliquots were withdrawn and diluted into 1 mL of reaction medium containing E2−E3 subcomplex (ratio E1:E2−E3 was 1:5) and all components required for assay of the PDH complex activity (see Methods). The reaction was initiated by the addition of 0.10 mM CoA.

with ThTTDP for 50 min at 25 °C, resulting in residual activities of 35% (I); 42% (II); and 8.6% (III). Next, ThDP (1 mM) and DTT (5 mM) were added and with a further 10 min of incubation, the residual activities were 71% (I), 61% (II), and 23% (III), indicating partial reactivation of the enzymes. However, after an additional 90 min of incubation the activity did not change significantly. Finally, there was no restoration of activity even after 15 h of incubation at 4 °C. A plausible explanation of the observations may be the formation of a disulfide bond between ThTTDP and C259, since addition of saturating concentrations of DTT led to partial restoration of the activity. In a model attempting to mimic such a reaction, thiamin 2-thiothiazolone was added to a solution of either KSH or the mercaptide form of 2-mercaptoethanol, resulting in a very fast reaction that did not take place with 3,4,5-trimethyl-2-thiothiazolone alone. So far, the product is unidentified. While the reaction did not take place at C2 according to ^{13}C NMR, the experiment

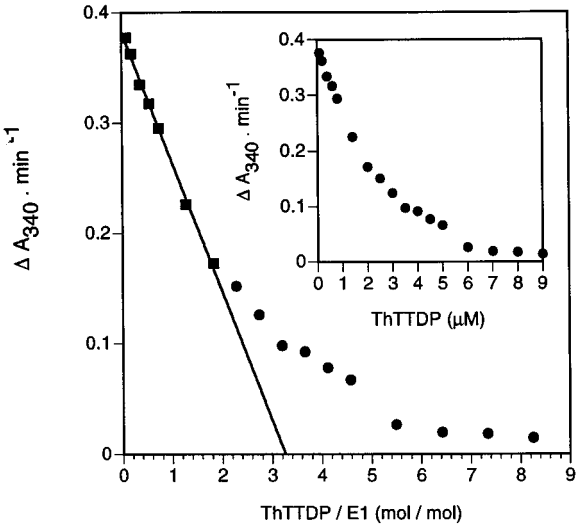

FIGURE 7: Stoichiometry of ThTTDP binding to parental E1. The parental E1 (1.09 μM) with tightly bound residual ThDP (2.99%) as determined according to the PDHc assay in the absence of additional ThDP and $MgCl_2$ was incubated in 50 mM KH_2PO_4 buffer (pH 7.0) with 0.2−50 μM ThTTDP and 2 mM $MgCl_2$ in a total volume of 0.1 mL at 25 °C. After 40 min of incubation, 10 μL aliquots were withdrawn and diluted into 0.250 mL of a reaction mixture containing E2−E3 subcomplex (ratio of E1:E2−E3 was 1:5) and all components required for assay of the PDH complex activity. The reaction was initiated by the addition of 0.13 mM CoA. Inset: Dependence of the steady-state rates for parental E1 on the concentration of ThTTDP (residual tightly bound ThDP = 2.99%).

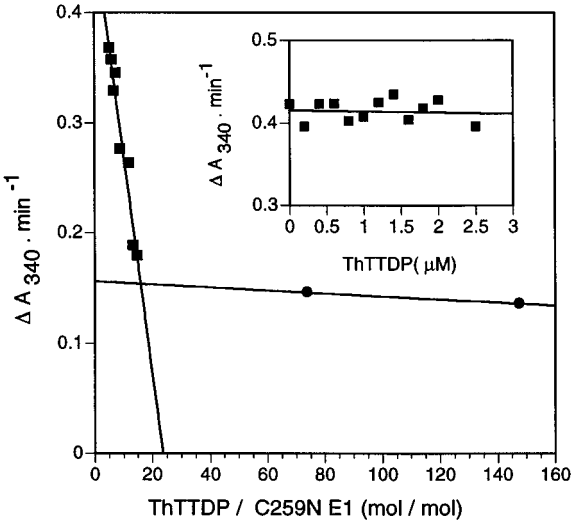

FIGURE 8: Stoichiometry of ThTTDP binding to the C259N variant of E1. The C259N variant of E1 (0.679 μM) was incubated in 50 mM KH_2PO_4 buffer (pH 7.0) with 0.2−100 μM ThTTDP and 2 mM $MgCl_2$ in a total volume of 0.1 mL at 25 °C. After 40 min of incubation , 10 μL aliquots were withdrawn and diluted into 0.250 mL of reaction mixture containing E2−E3 subcomplex (ratio E1:E2−E3 was 1:5) and all components required for assay of the PDH complex activity. The reaction was initiated by the addition of 0.13 mM CoA. Inset: Dependence of the steady-state rates for the C259N variant of E1 on the concentration of ThTTDP.

does raise the possibility of a reaction taking place between the inhibitor and C259.

DISCUSSION

It was shown by Schwartz and Reed (*5*) that isolated E1 component and E1−E2 subcomplex are inactivated by the

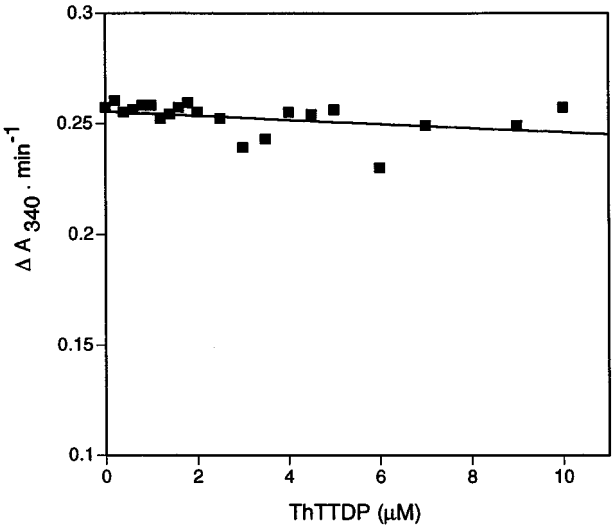

FIGURE 9: Dependence of the steady-state rates for the C259S variant of 1-lip PDHc on the concentration of ThTTDP. The C259S variant of 1-lip PDHc (0.67 mg/mL) was incubated in 50 mM KH_2PO_4 buffer (pH 7.0) with 0.2−10 μM ThTTDP and 2 mM $MgCl_2$ in a total volume of 0.1 mL at 25 °C. After 40 min of incubation, 10 μL aliquots were withdrawn and diluted into 0.250 mL of a reaction mixture containing all components required for assaying PDH complex activity. The reaction was initiated by the addition of 0.13 mM CoA.

organomercurials *p*-mercuribenzoate and *p*-mercuribenzenesulfonate. Complete inactivation of E1 apoenzyme occurred when approximately 1 SH group/monomer of E1 had reacted with mercurials. Adding ThDP (0.2 mM) in the presence of $MgCl_2$ (1.2 mM) afforded partial protection. Pyruvate alone offered no effective protection. A total of 2 SH groups/E1 monomer were titrated in the holoenzyme; however, only one of them was deemed important for activity (*5*).

In our laboratory, the activity of E1 resolved from PDHc was reduced to 55% by incubation with 0.3 mM methylmethanethiosulfonate or 5,5′-dithiobis(2-nitrobenzoic acid) at 25 °C when assayed with either 2,6-DCPIP as electron acceptor or as PDHc after reconstitution with an excess of E2−E3 subcomplex (data not shown). The time and concentration dependence of E1 inhibition by *p*-chloromercuribenzoate revealed that a 300-fold molar excess of modifier led to a total loss of enzyme activity (the residual activity was too small to detect after 30 min of incubation), suggesting that any SH group/groups important for activity must be deeply buried.

Elsewhere, it was shown that E1 component resolved from *E. coli* PDHc is inactivated by a 3−4-fold molar excess of *p*-hydroxymercuribenzoate (modification of 1 cysteine residue/monomer of E1 led to inactivation of the enzyme), yet under similar conditions, a 200-fold molar excess of DTNB or a 100-fold molar excess of iodoacetate did not affect the E1 activity. It was shown that mercurials less polar than *p*-hydroxymercuribenzoate inactivate the E1 component faster than *p*-hydroxymercuribenzoate did, indicating that hydrophobic interactions between the tested mercurials and the environment of an essential cysteine residue are responsible for the reactivity of the SH group (*29*).

To further elucidate the function of cysteines in E1, we have prepared all single cysteine variants. We have recently shown (*2*) that the C259S and C259N substitutions cause

partial reduction in the activity of 1-lip PDHc, to activities of 27% and 58%. The K_m for ThDP increased about 13-fold for the C259N variant of 1-lip PDHc compared to the parental 1-lip PDHc. The C259S variant of 1-lip PDHc was not saturated with even 5−10 mM ThDP. These experiments indicated that the cysteine residue in the putative ThDP binding fold is involved in cofactor binding (*2*). These observations are consistent with those reported earlier (*5*, *29*). The latter authors showed that the ESR spectrum of E1 modified by a spin-labeled analogue of *p*-hydroxymercuribenzoate is significantly changed upon addition of ThDP and Mg(II), indicating that binding ThDP·Mg(II) induces a conformational change close to an essential SH group.

In our studies four other cysteines were first replaced by serine by creating derivatives of pGS878 that express the C575S, C610S, C654S, and C770S E1 variants. All of these E1 variants could be reconstituted to PDH complexes with E2−E3 subcomplex that were 66% (C575S), 43% (C610S), 65% (C654S), and 90% (C770S) as active as that obtained with parental E1. The parental E1 and all variants with serine substitutions were inactivated in the presence of 0.5 mM fluoropyruvate. The residual activities after 35 min incubation at 25 °C were 12%, parental E1; 15%, C575S; 15%, C610S; 13%, C654S; and 12%, C770S. These substitutions clearly demonstrated that none of these cysteines is essential for activity or is involved in the inactivation by fluoropyruvate. We next converted three of the cysteines to alanines (C575, C610, and C654, whose conversion to serine led to a significant decrease in activity) and the remaining sixth C120 to alanine as well. These alanine variants were also active, as shown in Table 1, confirming that none of the five cysteine residues outside the putative ThDP fold is essential for E1 activity, irrespective of whether it was converted to serine or alanine.

The following conclusions could be drawn from detailed kinetic studies on all cysteine variants:

(1) All of the E1 variants could be successfully reconstituted with E2−E3 subcomplex and the corresponding PDHc activities were not significantly different from that observed with parental E1.

(2) None of the cysteine residues of E1 is essential for pyruvate binding according to the apparent K_m for cysteine variants of E1 (Table 1). The apparent K_m for pyruvate for the C259S variant of 1-lip PDHc is 5 times higher than for the parental 1-lip PDHc; however, for the C259N variant of 1-lip PDHc the K_m values are very close to those for the parental complex.

(3) All variants of E1 and the C259S and C259N variants of 1-lip PDHc are subject to allosteric inhibition by acetyl-CoA; however, this inhibition is less pronounced with reconstituted PDHc than with preformed complexes.

(4) The C259S and C259N variants of 1-lip PDHc and five variants of E1 with single cysteine substitutions were all inhibited by fluoropyruvate, indicating that none of the six cysteine residues in the E1 subunit is involved in the inactivation mechanism.

(5) On the basis of the kinetics of inactivation by 2-oxo-3-butynoic acid and the changes in SH group content concomitant with inactivation, it is likely that C575, and perhaps C770, reacts with 2-oxo-3-butynoic acid during the inactivation; they are most likely alkylated.

(6) Cys259, located in the ThDP binding fold, is important for ThTTDP binding. Gutowski and Lienhard (*19*) reported that thiamin 2-thiazolone diphosphate binds to *E. coli* E1 20 000 times more tightly than ThDP. It was proposed that this compound with a C2═O bond resembles the transition state formed upon decarboxylation of pyruvate in ThDP-dependent enzymes; hence they termed this compound a transition-state analogue. However, ThTDP binds to yeast transketolase much less tightly than to *E. coli* E1 (*30*); the K_i for ThTDP was 28 nM, compared to 0.5 nM for *E. coli* E1. On the basis of this analysis, it was concluded that ThTDP is not a transition-state analogue inhibitor of transketolase. The crystal structure of transketolase complexed with ThTDP further showed that ThTDP and ThDP bind in the same manner (*31*), indicating that there are no additional specific interactions between ThTDP and transketolase. Also, it was shown that for wheat germ pyruvate decarboxylase (*21*) the K_i for ThTDP is 2 μM, very similar to the K_m (6 μM) for ThDP. It was also found that ThTTDP and ThDP have similar affinities for apo-PDC (from which ThDP has been resolved) from brewer's yeast (*32*, *33*) and that ThTDP binds to the same site as ThDP in *E. coli* pyruvate oxidase (*34*). The K_d for ThTDP was 0.2 μM compared to the K_d of 6 μM for ThDP, indicating that in pyruvate oxidase ThTDP does not behave as a transition-state analogue.

A comparison of the ThDP-binding motifs of ThDP-dependent enzymes shows that *E. coli* E1 is unique in terminating in NCN, rather than NN (*4*). The data presented in this paper indicate that ThTTDP binding to *E. coli* E1 is virtually abolished in the C259 variants, ruling out a prior explanation that nonpolar interactions in the active site of *E. coli* E1 were responsible for the tight binding observed with this compound (*31*). We therefore conclude that the very strong inhibition of *E. coli* E1 by these two analogues does not represent a general "transition-state binding" model for all of these ThDP enzymes; rather it is the result of a highly specific interaction between the enzyme active center and these compounds.

Finally, on the basis of our results, we conclude that the mechanism of inactivation of this enzyme by fluoropyruvate and 2-oxo-3-butynoic acid (probably bromopyruvate as well) remains unresolved.

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