Inactivation of the Pyruvate Dehydrogenase Complex of *Escherichia coli* by Fluoropyruvate†

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ABSTRACT: The pyruvate dehydrogenase complex (PDH complex) of *Escherichia coli* and its pyruvate dehydrogenase component (E_1) are rapidly inactivated by low concentrations of fluoropyruvate in a thiamin pyrophosphate (TPP) dependent process. The inactivation rates for the PDH complex and for its E_1 component are similar. Pyruvate protects the PDH complex and the E_1 component against inactivation by fluoropyruvate. Dihydrolipoamide protects the E_1 component from inactivation. TPP is not covalently bound to the PDH complex or to the E_1 component by the inactivating reaction. When [^{14}C]fluoropyruvate is used to inactivate the PDH complex, ^{14}C remains bound to the complex after gel filtration. This bound radioactivity is cleaved from the protein by NH_2OH, ^-OH, and $NaBH_4$ but not by dilute acid. When released by ^-OH, greater than 90% of the ^{14}C cochromatographs with acetate on DEAE-Sephadex. When released by $NaBH_4$, and ^{14}C is recovered as [^{14}C]ethanol. Colorimetric analysis for sulfhydryl groups on the native E_1 component and the inactivated E_1 component, using 5,5′-dithiobis(2-nitrobenzoate), reveals that complete inactivation results from covalent modification of 1.37 ± 0.03 sulfhydryl residues. Fluoropyruvate is known to generate acetyl-TPP at the active site of E_1. The available evidence indicates that acetylation of a sulfhydryl group by acetyl-TPP at the active site of the E_1 component inactivates the enzyme.

The pyruvate dehydrogenase complex of *Escherichia coli* is a multienzyme complex that catalyzes the decarboxylation and dehydrogenation of pyruvate with concomitant acetylation of CoASH[1] and reduction of NAD^+ according to the stoichiometry shown in eq 1. The complex is composed of three en-

$$CH_3COCO_2^- + CoASH + NAD^+ \rightarrow CO_2 + CH_3COSCoA + NADH \quad (1)$$

zymes: pyruvate dehydrogenase (E_1), the thiamin pyrophosphate (TPP) dependent component; dihydrolipoyl transacetylase (E_2), which contains covalently bound lipoic acid; and dihydrolipoyl dehydrogenase (E_3), a flavoprotein which contains noncovalently bound FAD. The E_1 component of the PDH complex also catalyzes the decomposition of fluoropyruvate to CO_2, acetate, and fluoride anion according to eq 2 (Leung & Frey, 1978). Fluoropyruvate is not a substrate

$$FCH_2COCO_2^- + H_2O \rightarrow CO_2 + CH_3CO_2^- + F^- + H^+ \quad (2)$$

for the PDH complex in the overall reaction it catalyzes (eq 1). The decomposition of fluoropyruvate is strictly TPP dependent and is thought to proceed by the mechanism shown in Scheme I (Leung & Frey, 1978). The evidence in support of this mechanism includes the following: (i) fluoride anion and acetate are formed with a stoichiometry of 1:1; (ii) when the reaction is carried out in ^3H_2O, [^{3}H]acetate is recovered; and (iii) the reaction is strictly dependent on the presence of E_1 and MgTPP.

In addition to being an unusual substrate for the pyruvate dehydrogenase component, fluoropyruvate also inactivates E_1 in a TPP-dependent fashion (Leung, 1977). The mechanism of this inactivation is the subject of this paper. Contrary to a report in the literature (Bisswanger, 1981), we have found that fluoropyruvate is an irreversible inactivator of both the PDH complex and the E_1 component of the complex. This inactivation results from acetylation of a thiol residue at or near the active site of the E_1 component. The evidence indicates that the acetylating species is acetyl-TPP, which is formed within the active site following fluoride elimination (Scheme I).

Experimental Procedures

Chemicals. Urea was purchased from Merck and crystallized from ethanol prior to use. Ethanol and triethylamine were distilled prior to use. DEAE-Sephadex A-25 was prepared and converted to the formate form as previously described (Flournoy & Frey, 1986). Sephadex G-50 (fine) was allowed to swell in 50 mM KP_i buffer at pH 8.0 for 48 h at 4 °C prior to use.

Enzymes. The PDH complex and the pyruvate dehydrogenase component (E_1) were prepared and stored as previously described (Reed & Willms, 1966; Flournoy & Frey, 1986). Preparations of the E_1 subunit showed no detectable activity when assayed for PDH complex activity (eq 1) as described below, even when 135 μg of the E_1 component was used in the assay.

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[1] Abbreviations and common names: acetyl-TPP, 2-acetylthiamin pyrophosphate; bicine, *N,N*-bis(2-hydroxyethyl)glycine; CoASH, coenzyme A; DHLA, D,L-dihydrolipoamide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); E_1, the pyruvate dehydrogenase component of the PDH complex; E_2, the dihydrolipoyl transacetylase component of the PDH complex; E_3, the dihydrolipoyl dehydrogenase component of the PDH complex; FAD, flavin adenine dinucleotide, oxidized form; fluoroacetofluorolactate, 4-fluoro-2-(fluoromethyl)-2-hydroxy-3-oxobutanoic acid; fluoropyruvate, 3-fluoro-2-oxopropanoic acid; [^{14}C]fluoropyruvate, [1,2-$^{14}C_2$]-3-fluoro-2-oxopropanoic acid; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; PDH complex, pyruvate dehydrogenase complex; TCA, trichloroacetic acid; TEAF, triethylammonium formate; TPP, thiamin pyrophosphate.

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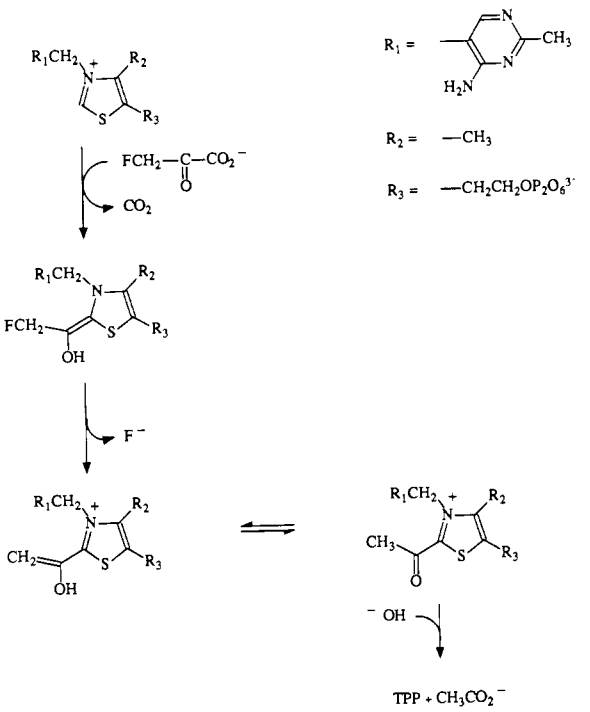
Scheme I



The $E_2 \cdot E_3$ subcomplex was prepared according to the procedure of Reed and Willms (1966), which was modified as follows. After elution of the E_1 subunit from calcium phosphate gel–cellulose, the column was washed with ethanolamine–P_i buffer, pH 9.5, until the absorbance at 280 nm was equal to or less than 0.005. The column was then developed with 4% $(NH_4)_2SO_4$, dissolved in 0.1 M KP_i, pH 7.5. The fractions containing the $E_2 \cdot E_3$ subcomplex were collected and centrifuged at 130000*g* (38 000 rpm), the supernatant fluid was discarded, and the pellets were dissolved in 50 mM KP_i buffer, pH 7.0. Preparations of the $E_2 \cdot E_3$ subcomplex showed 2–5% of the activity (eq 1) of the intact complex when assayed as described by Maldonado et al. (1972). The $E_2 \cdot E_3$ subcomplex was stored at −70 °C until used.

Acetate kinase and lactate dehydrogenase were purchased from Boehringer Mannheim and Sigma, respectively.

Substrates and Cofactors. Coenzyme A, thiamin pyrophosphate, NAD^+, NADH, and sodium pyruvate were all purchased from Sigma. Sodium fluoropyruvate was purchased from Aldrich. Dihydrolipoamide and [^{14}C]fluoropyruvate were synthesized as previously described (Flournoy & Frey, 1986).

Analytical Methods. Concentrations of pyruvate and fluoropyruvate in solutions were measured with lactate dehydrogenase and NADH. Acetate was measured by the method of Rose et al. (1954). Dihydrolipoamide in solutions was measured spectrophotometrically by reaction with DTNB, assuming an extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm (Ellman, 1959). The concentration of lipoamide was determined by measurement at 330 nm, assuming an extinction coefficient of 150 M^{-1} cm^{-1} (Reed, 1966). The concentrations of other coenzymes were determined spectrophotometrically with extinction coefficients (M^{-1} cm^{-1}) of 7700 at 269 nm for TPP (Goedde et al., 1962), of 16 000 at 260 nm for CoASH (Decker, 1959), of 18 000 at 260 nm for NAD^+ (Siegel et al., 1959), and of 6200 at 340 nm for NADH (Horecker & Kornberg, 1948). The concentration of DTNB was determined by treatment with excess cysteine and by measurement of the absorbance at 412 nm. The molar concentration of DTNB was assumed to be half the concentration of 2-carboxy-4-(nitrothio)phenolate anion formed and measured at 412 nm, assuming an extinction coefficient of 13.6 mM^{-1} cm^{-1} (Ellman, 1959).

The PDH complex was assayed for its activity in producing NADH (eq 1) under the conditions of Maldonado et al. (1972). To assay the E_1 component, it was first incubated with the $E_2 \cdot E_3$ subcomplex as described below to reconstitute the PDH complex. The reconstituted complex was then assayed for its activity in producing NADH as described above for the native complex.

Protein concentrations of the $E_2 \cdot E_3$ subcomplex, the E_1 subunit, and ^{14}C-labeled E_1 were estimated by the method of Lowry (Cooper, 1977). Protein concentrations of the purified PDH complex were determined by measurements at 280 nm, by use of a value of 1.0 for $E^{1\%}$ determined by Speckhard (1974), who used standard solutions of PDH complex whose concentrations had been established by dry weight measurements. The concentrations of solutions of ^{14}C-labeled PDH complex were determined as for native complex.

In radiochemical assays samples to be counted were placed in scintillation vials and the volumes bought to 1 mL with water. Aquasol, 15 mL, was added, and the samples were placed in the dark a minimum of 15 min prior to counting. Samples were counted on a Beckman LS-1000 liquid scintillation spectrometer.

All UV–vis spectroscopy was performed on a Cary 118C, a Hitachi 100-80 A, or a Unicam SP 1800 spectrophotometer. Fluorescence was measured on an Aminco-Bowman spectrofluorometer.

Assay of TPP as Thiochrome Pyrophosphate. Standard solutions of TPP were prepared and their concentrations established spectrophotometrically as described above. TPP in these solutions or from enzymatic reaction mixtures was converted to thiochrome pyrophosphate as follows (Wostman & Knight, 1960). The test sample of TPP was placed in 1.0 mL of 50 mM sodium bicinate (or KP_i) buffer, pH 8.1. Then, 0.1 mL of $K_3Fe(CN)_6$ [9 mM in 15% (w/v) NaOH] was added and the reaction mixture allowed to sit at room temperature for 8 min. At this time the reaction was quenched by the addition of 33 μL of a 0.03 M solution of cysteine. The visible spectrum of the reaction mixture showed a new band at 360 nm with an extinction coefficient of approximately 11.9 mM^{-1} cm^{-1} (if 100% conversion is assumed). Formation of this new band is complete in 2 min when the initial TPP concentration is 46 μM. The increase in absorbance at 360 nm is linear with increasing TPP in the test sample up to approximately 45 nmol. Under the conditions existing at the end of the reaction, the color is stable for at least 4 h at room temperature (ca. 22 °C). The fluorescence of the thiochrome pyrophosphate was measured at 456 nm (excitation at 360 nm). The relative fluorescence increases linearly with respect to the TPP concentration. FAD did not interfere in the assays.

Inactivation of the Pyruvate Dehydrogenase Complex and the E_1 Component by Fluoropyruvate. The pyruvate dehydrogenase complex or its E_1 subunit was incubated with $MgSO_4$, TPP, fluoropyruvate, and KP_i or sodium bicinate buffer as described in the figure legends. All inactivations were initiated by the addition of fluoropyruvate. The activity at zero time was determined by removing and assaying an aliquot of enzyme prior to the addition of fluoropyruvate. This value was defined as 100% activity, and all subsequently measured activities were related to it as a percentage or as ln (percent of the activity at the zero time). At the times indicated in the figures, 10-μL samples were removed and diluted into 1.0 mL of cold KP_i buffer, 50 mM, at pH 8.1. In the case of the pyruvate dehydrogenase complex, 10 μL of this solution was

assayed directly for NADH activity as described above, in a reaction mixture whose final volume was 1.0 mL. Thus, in the solution in which activity was measured, fluoropyruvate was diluted by a factor of 1:10 000.

In the inactivation of the E_1 component, 10 μL was removed from the initial reaction mixture and diluted into 1.0 mL of cold KP_i buffer (50 mM, pH 8.1) which contained 40 μg of $E_2 \cdot E_3$ subcomplex. Then, 20 μL of this material was removed and assayed for pyruvate dehydrogenase activity (eq 1) as described above.

Preparation of ^{14}C-Labeled Pyruvate Dehydrogenase Complex. [^{14}C]PDH complex was prepared by incubating the PDH complex with [^{14}C]fluoropyruvate (0.1 mM) in sodium bicinate buffer (50 mM, pH 8.0) in the presence of 1 mM MgTPP. After the activity had dropped by 80–90% the solution was centrifuged at 130000*g* (38 000 rpm) for 3–4 h. The supernatant was removed and the pellet dissolved in 50 mM KP_i buffer, pH 7.0. This protein solution was then applied to a column of Sephadex G-50 (fine) (1 × 48 cm) which had been equilibrated with 50 mM KP_i buffer at pH 7.0 and eluted with the same buffer. Fractions were collected, and those containing [^{14}C]PDH complex were identified by measurements of absorbance at 280 nm, radiochemical assays, and assays for residual enzymatic activity.

Preparation of ^{14}C-Labeled E_1 Component. [^{14}C]E_1 was prepared by inactivation of the E_1 component with [^{14}C]-fluoropyruvate (0.05 mM) in sodium bicinate buffer (50 mM, pH 8.0) in the presence of 1 mM MgTPP. After the activity had dropped by 80–90%, the reaction mixture was brought to 0.8 saturation with $(NH_4)_2SO_4$. The mixture was stirred at room temperature for 15 min and then centrifuged at 18000*g* (14 000 rpm) for 10 min. The supernatant fluid was removed and the pellet dissolved in KP_i buffer (50 mM, pH 7.0). This protein solution was applied to a column of Sephadex G-50 (fine) (1 × 43 cm), equilibrated and eluted with 50 mM KP_i buffer (pH 7.0). Fractions of approximately 1.8 mL were collected and those containing [^{14}C]E_1 identified by measurements of absorbance at 280 nm, radioactivity content, and residual enzymatic activity.

Analysis of Native and Fluoropyruvate-Inactivated E_1 for Sulfhydryl Groups. Native or fluoropyruvate-inactivated E_1, in 2 mM KP_i at pH 7.0, was diluted to 1 mg/mL with a 10.44 M solution of urea that had been prepared in 50 mM KP_i at pH 8.1. The final urea concentration was approximately 8.1 M, and the final buffer concentration was approximately 39 mM. This mixture was allowed to sit at room temperature (ca. 24 °C) for 5 min. At this time excess DTNB from a 0.58 mM stock solution was added and the absorbance at 412 nm determined. The concentration of free sulfhydryl groups was determined from the extinction coefficient of 13.6 mM^{-1} cm^{-1} at 412 nm for the 2-carboxy-4-(nitrothio)phenolate anion. The calculations of the sulfhydryl content in the inactivated samples were corrected for their degree of inactivation, which had been determined as described above.

Reduction of [^{14}C]PDH Complex by $NaBH_4$. [^{14}C]PDH complex (1.2 mg/mL) was incubated with urea (approximately 8 M) and stirred for 30 min. This solution was added to a flask containing 24 mg of solid $NaBH_4$ and stirred magnetically. After 30 min 960 μL of 1 N HCl was added and the mixture stirred an additional 5 min. The pH was adjusted to 6.0 with NaOH and 20 mL of 95% ethanol added. The ethanol was isolated by distillation and collected as the fraction boiling between 77 and 80 °C. The distillate was allowed to stand over 3-Å sieves for 24 h; 1.0 mL was then removed and counted to determine the specific activity, which remained constant upon redistillation of the [^{14}C]ethanol.

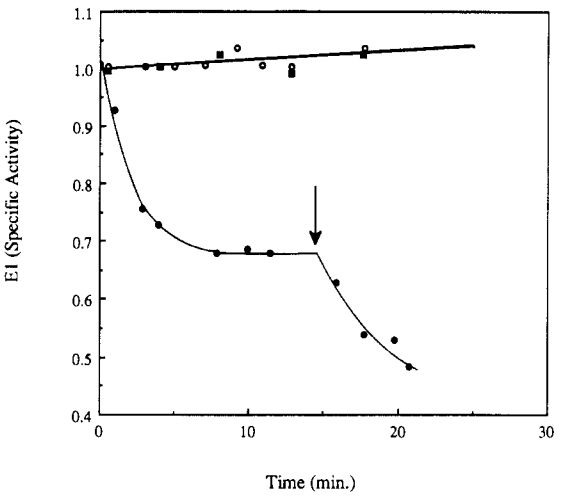


FIGURE 1: Inactivation of the PDH complex by fluoropyruvate. The PDH complex (11 mg/mL) was incubated at 29 °C with TPP (0.2 mM), $MgSO_4$ (1 mM), KP_i buffer (50 mM, pH 6.75), and fluoropyruvate (1 mM). At the indicated times aliquots (0.05 mL) of the reaction mixture were withdrawn and diluted into 0.45 mL of cold buffer. One-tenth milliliter of each of the diluted enzyme solutions was assayed for E_1 activity (●). After 15 min (arrow) additional fluoropyruvate was added to the reaction mixture. In our control experiments the enzymatic activity was stable when TPP (○) or fluoropyruvate (■) was omitted from the reaction mixture. Adapted from Leung (1977).

TPP Binding Studies. The PDH complex (2 mg/mL) was incubated with $MgSO_4$, TPP, and fluoropyruvate in KP_i buffer (50 mM, pH 8.0) in a final volume of 0.65 mL. All components, except fluoropyruvate, were incubated with the protein for 10 min at room temperature prior to initiating the inactivation by addition of fluoropyruvate. From this reaction mixture 10-μL samples were removed and diluted into 2.0 mL of cold KP_i buffer (50 mM, pH 8.0) from which 10 μL was then removed for determination of residual enzymatic activity. At the same time, 100-μL samples were removed and delivered into microcentrifuge tubes containing 10 μL of 20% (w/v) TCA. These samples were centrifuged in a Brinkmann Eppendorf Centrifuge 3200, and the supernatant fluid was removed. The precipitated protein was washed with 50 mM KP_i buffer, pH 8.0 (2 × 0.45 mL), and centrifuged. The supernatant fluids were all combined. Analysis for TPP, by conversion to thiochrome pyrophosphate, was performed on these samples as described above.

The pyruvate dehydrogenase component (1 mg/mL) was incubated as above except that the fluoropyruvate concentration was 0.05 mM in sodium bicinate buffer (50 mM, pH 8.0). At the indicated times 10 μL was removed and delivered into a test tube containing 1.0 mL of cold KP_i buffer (50 mM, pH 8.0); the residual enzymatic activity of this protein was determined as described above for the assay of E_1. At the same time, 100 μL was removed and delivered into a microcentrifuge tube containing 10 μL of 20% TCA and centrifuged, and the precipitate was washed as described above for the PDH complex. The supernatant fluids were then collected and assayed for TPP by its conversion to thiochrome pyrophosphate.

RESULTS

Kinetics of Inactivation by Fluoropyruvate. When the PDH complex of *Escherichia coli* is incubated with fluoropyruvate, Mg^{2+}, and TPP, the enzymatic activity (eq 1) decreases with time (Figure 1). The inactivation kinetics is complex, both for the inactivation of the E_1 component and for the inactivation of the PDH complex. Several factors contribute to this

Table I: Dependence of Inactivation Rate on Initial Concentration of Fluoropyruvate[a]

[fluoropyruvate] (mM)	k_{inact} (min^{-1}) PDH complex	k_{inact} (min^{-1}) E_1 component
0.008	0.064	
0.010		0.066
0.015	0.11	
0.020		0.15
0.022	0.11	
0.025	0.14	
0.030		0.19
0.034	0.20	
0.040		0.21
0.050	0.13	0.13
0.062	0.14	
0.080		0.083
0.10	0.10	0.080
0.20	0.049	
0.30	0.044	
0.40	0.028	
0.60	0.027	

[a] The reaction mixtures consisted of the PDH complex or the E_1 component (1 mg/mL), TPP (1 mM), $MgSO_4$ (1 mM), sodium bicinate buffer (50 mM, pH 8.0), and fluoropyruvate ranging in concentration from 0.008 to 0.6 mM. Aliquots of each reaction mixture were withdrawn at appropriate times, quenched by dilution into cold buffer, and assayed for residual enzymatic activity by the procedures described under Experimental Procedures. The activity versus time data for each reaction mixture were plotted on semilog graph paper for the initial one to two half-times, and the slopes were used to calculate the observed first-order rate constants for inactivation. The rates constants for inactivation are tabulated here as a function of the initial concentration of fluoropyruvate.

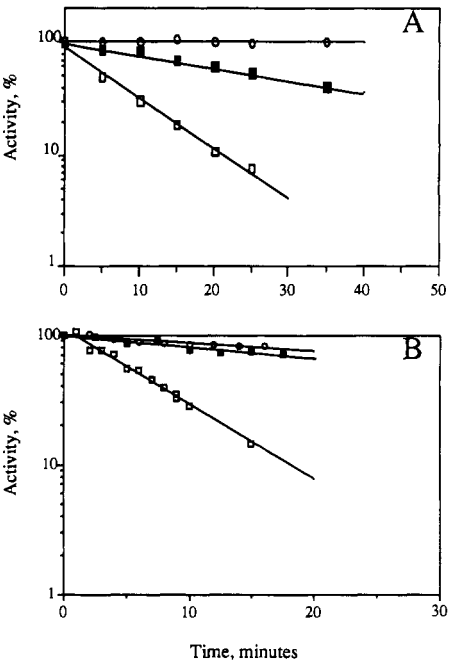


FIGURE 2: TPP dependence of and substrate protection from inactivation by fluoropyruvate. The reaction mixtures consisted of the PDH complex or the E_1 component (1 mg/mL), TPP (1 mM), $MgSO_4$ (1 mM), fluoropyruvate as indicated below, and sodium bicinate buffer (50 mM, pH 8.0) in the presence (■) or absence (□) of pyruvate. The dependence on TPP was examined in control reaction mixtures from which TPP was excluded (○). At the indicated times aliquots of the reaction mixtures were withdrawn and assayed for residual enzymatic activity as described under Experimental Procedures. Panel A gives the data obtained in the inactivation of the PDH complex with 0.1 mM fluoropyruvate, and panel B gives the corresponding data for the inactivation of the E_1 component with 0.05 mM fluoropyruvate.

complexity. First, fluoropyruvate is consumed by its reaction as an unusual substrate in eq 2, and probably also in the carboligase reaction discussed below, so that its concentration decreases during an inactivation experiment. At high concentrations of fluoropyruvate the inactivation does not approach completion but reaches a plateau and, upon readdition of fluoropyruvate, approaches a lower plateau. This is shown in Figure 1. Second, the initial inactivation rate (k_{inact}) increases with increasing fluoropyruvate concentration at low concentrations and then decreases with increasing fluoropyruvate concentration at high concentrations. This is shown in Table I, where the initial inactivation rate constants are tabulated as a function of the initial concentration of fluoropyruvate.

Table I leads to two conclusions. First, the inactivation effects on the E_1 component and the PDH complex in the initial phase almost certainly reflect the same molecular process, since the rates are the same within error. Second, the initial inactivation rate increases proportionally to increasing concentration of fluoropyruvate from 0.01 (k_{obs} = 0.06 min^{-1}) to 0.03 mM (k_{obs} = 0.2 min^{-1}), but the decline in inactivation rate at higher concentrations means that some other interaction intervenes to protect the enzyme. The results in Table I show that inactivation takes place at a concentration of fluoropyruvate as low as 0.008 mM. At this concentration fluoropyruvate is stoichiometrically equal to the E_1 active sites under the conditions of Table I.

The apparently anomalous kinetics at high fluoropyruvate concentrations in Figure 1 and Table I can be explained by the carboligase activity of E_1 (Kubasik et al., 1972). In the carboligase reaction a molecule of pyruvate is decarboxylated to hydroxyethylidene-TPP at the E_1 site, and this intermediate reacts with a second molecule of pyruvate to form acetolactate (see Discussion). This reaction comes into play at high concentrations of the keto acid, in this case fluoropyruvate; and the carboligase product does not irreversibly inhibit the enzyme. Thus the residual activity reaches a plateau value in Figure 1, where the fluoropryvate concentration is 1 mM. At low concentrations of fluoropyruvate, <0.1 mM, the PDH complex and E1 undergo irreversible inactivation. All of the following experiments refer to inactivation at low concentrations of fluoropyruvate.

Fluoropyruvate is known to bind the first enzyme (E_1) in the PDH complex and undergo TPP-dependent decomposition according to eq 2. Steps leading to inactivation may have steps in common with this reaction. If so, fluoropyruvate should bind in competition with pyruvate, and inactivation should be TPP dependent. Figure 2A shows the TPP dependence of the inactivation process and also the protective effect of pyruvate, indicating that fluoropyruvate binds at the active site and interacts with TPP. The inactivation of the E_1 component shows a similar dependence on TPP and protection by pyruvate (Figure 2B).

Labeling of E_1 by [^{14}C]Fluoropyruvate. Figures 1 and 2 suggest that inactivation of the complex results from inactivation of the E_1 component. If so, and if inactivation leads to irreversible binding of carbon from fluoropyruvate to the enzyme, then [^{14}C]fluoropyruvate should label only E_1 within the PDH complex. When the PDH complex, or its E_1 component, is incubated with [^{14}C]fluoropyruvate, both enzymes lose activity and become ^{14}C labeled. This label is retained after gel filtration through columns of Sephadex G-50 (data not shown). Separation of the E_1 component of the complex from the E_2·E_3 subcomplex shows that the ^{14}C label migrates predominantly with the E_1 component of the complex. Figure 3 presents the results obtained in such an experiment, in which 90% of the ^{14}C migrated with the E_1 component and the re-

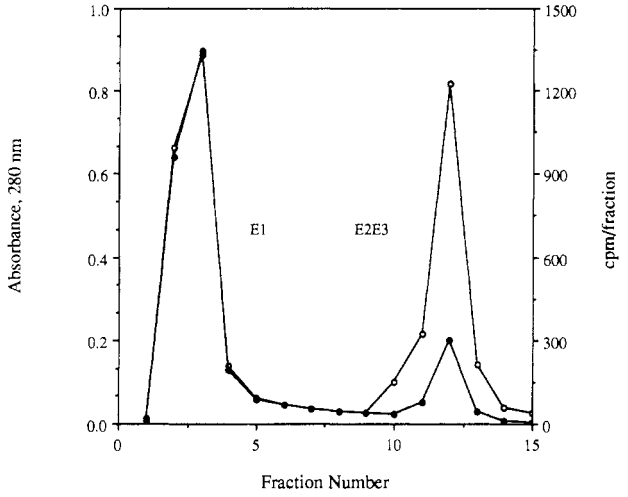


FIGURE 3: Separation of [^{14}C]E_1 from [^{14}C]PDH complex. The PDH complex (10 mg/mL) prepared by inactivation with [^{14}C]fluoropyruvate in the presence of MgTPP (see Experimental Procedures) was adjusted to pH 9.8 by addition of 0.16 M ethanolamine. The solution was applied to the top of a calcium phosphate gel–cellulose (1 × 4.5 cm) and washed through with ethanolamine phosphate buffer at pH 9.8 as described by Reed and Willms (1966). Nine fractions (2 mL each) were collected, and the column was then eluted with 4% $(NH_4)_2SO_4$ dissolved in 0.1 M KP_i buffer at pH 7.5. The absorbance at 280 nm (○) and the total radioactivity (●) for each fraction were determined.

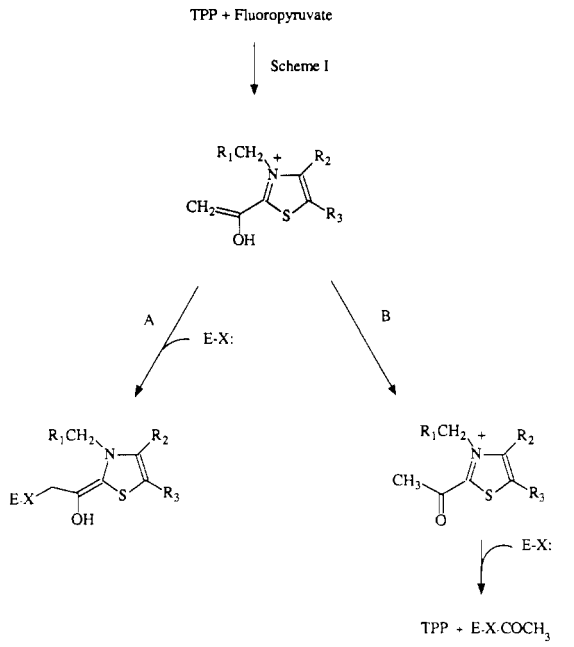
maining 10% migrated with the E_2·E_3 subcomplex.

It is very unlikely that the residual radioactivity in the E_2·E_3 subcomplex represents labeling of E_2 or E_3, since the resolution of E_1 by the procedure of Figure 3 is always incomplete, so that the E_2·E_3 subcomplex is always contaminated by E_1. Analysis of E_1 and the E_2·E_3 subcomplex from Figure 3 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, which separates E_1, E_2, and E_3, confirmed the contamination of the E_2·E_3 subcomplex by E_1 and showed E_1 to be free of the other two proteins.

Mechanism of Inactivation by Fluoropyruvate. We can consider two general mechanisms to account for inactivation of the E_1 component on the basis of the known chemistry of TPP and the reactivity of fluoropyruvate with E_1 as defined in eq 2 and Scheme I (Leung & Frey, 1978). These mechanisms are shown in Scheme II. In both mechanisms fluoropyruvate binds to the E_1 active site in a manner analogous to that of pyruvate. It then reacts by addition of the carbon-2 anion of TPP, by analogy with the reaction of pyruvate as a substrate, to form fluorolactyl-TPP, which is analogous to lactyl-TPP formed by pyruvate.

Fluorolactyl-TPP then undergoes decarboxylation to 2-fluoro-1-hydroxyethylidene-TPP, which is analogous to 1-hydroxyethylidene-TPP, the decarboxylated intermediate derived from the reaction of pyruvate. Up to this point the reactions of fluoropyruvate are strictly analogous to those of pyruvate. However, the reaction of fluoropyruvate diverges from that of pyruvate at this point by the elimination of fluoride anion from 2-fluoro-1-hydroxyethylidene-TPP. This elimination is favored by the stability of fluoride as a leaving group and by the reactivity of the intermediate, 2-fluoro-1-hydroxyethylidene-TPP, as a resonance-stabilized carbanionic equivalent. The immediate product of fluoride elimination is enolacetyl-TPP, which can be expected to ketonize, albeit at an unknown rate. Two reasonable reactions of this intermediate could lead to irreversible inactivation of E_1. It is possible that an enzymic nucleophile might undergo a Michael-type addition to carbon-2 of enolacetyl-TPP. This would generate a stable bond between the enzymic nucleophile and carbon-2

Scheme II



of 1-hydroxyethylidene-TPP (pathway A). Alternatively, enolacetyl-TPP might undergo ketonization to acetyl-TPP. This is a presumed intermediate in the reaction of fluoropyruvate as an alternative substrate (Scheme I). Acetyl-TPP could then react by either of two routes, hydrolysis by water to acetate and TPP or acetyl group transfer to an enzymic nucleophile to form an acetyl-enzyme (pathway B).

Both pathways in Scheme II postulate the existence of an enzymic nucleophile that is chemically modified by the inactivation process. However, the two products resulting from modification of this nucleophile differ chemically and can be distinguished by chemical analysis. The enzymic adduct obtained by pathway A is electronically analogous to 1-hydroxyethylidene-TPP and should be protonated upon acid denaturation of the protein. Therefore, the TPP would be covalently bound to the enzyme by the bridging hydroxyethyl group following acid denaturation. If mechanism B is operative, the TPP is released from the acetyl group in the process of modifying the nucleophile and will be released from the enzyme upon acid denaturation.

To determine whether pathway A or B is involved in the inactivation, we subjected both the PDH complex and the E_1 component separately to inactivation by fluoropyruvate and analyzed the reaction mixtures for residual activity and loss of TPP. TPP was assayed as thiochrome pyrophosphate after precipitation of the protein by addition of TCA, as described under Experimental Procedures. The reaction mixtures consisted of the PDH complex (2 mg/mL) or the E_1 component (1 mg/mL), TPP (0.01 mM), $MgSO_4$ (1 mM), fluoropyruvate (0.16 mM with PDH complex or 0.05 mM with E_1), and 50 mM buffer at pH 8.0 (KP_i for PDH complex or sodium bicinate for E_1). The enzyme activities decreased in both experiments by 70–80% during 20 min. Assays for TPP at 2-min intervals showed that the TPP in supernatant fluids from acid-precipitated samples reflected the TPP contents of the reaction mixtures and remained constant throughout the course of inactivation. In these experiments the amounts of TPP present were stoichiometrically equivalent to the amount of E_1, so that 50% inactivation should have resulted in 50% less TPP released upon acid denaturation if the coenzyme had been covalently bound to the protein. Inasmuch as inactivation had no effect on the amount of TPP released, the coenzyme could

not have been covalently bound in the manner illustrated by pathway A in Scheme II.

The exclusion of pathway A by the above experiment does not establish that pathway B in Scheme II is the inactivation mechanism. The inactivating species in pathway B is acetyl-TPP, which acetylates a nucleophilic group to form an inactive acetyl-enzyme. The identification of the inactive protein as an acetyl-enzyme would support pathway B as the mechanism of inactivation. In an acetylated enzyme the nucleophilic acetyl acceptor might be a sulfhydryl or an amino group. It is unlikely to be a carboxyl or imidazole group because the product of such a reaction would be an acid anhydride or acetylimidazole, which are unlikely to be stable in aqueous solution under the reaction conditions and product isolation conditions of our experiments. In particular, the protein-bound ^{14}C is not released by denaturation of the protein by either acid or urea, which rules out both of these possibilities.

Identification of the Inactivated Enzyme as an Acetyl Thioester. To characterize the inactivated enzyme, we investigated its chemical properties. We examined the possibility that it might be a thioester by testing the ability of hydroxylamine to remove the acetyl group from the ^{14}C-labeled protein. Incubation of the ^{14}C-labeled PDH complex with 1.0 M NH_2OH at pH 8.0 removed 65% of the ^{14}C after 60 min and 73% after 120 min, as revealed by gel filtration experiments [0.4-mL aliquots filtered through small columns (0.7 cm × 17 cm) of Sephadex G-50]. Additional data were obtained for reaction times of 20, 40, and 90 min and plotted with the above on semilog graph paper, which showed that the release of ^{14}C from the protein is a first-order process with a half-time of 50 min at room temperature. A thioester would be cleaved by NH_2OH under these conditions to form acetohydroxamate, but an acylamide would not react. Therefore, the inactive enzyme could not be an acetamide but could be an acetyl thioester.

The radioactivity in the ^{14}C-labeled enzyme is also removed by hydroxide ions. Incubation of ^{14}C-labeled PDH complex with base (NaOH, pH 12.6) for 0.5 h at room temperature quantitatively releases the radioactivity from the protein. Subsequent chromatography of the released radioactivity shows that it binds to DEAE-Sephadex (formate form) and that greater than 90% of the ^{14}C coelutes with acetate when chromatographed by the method described by Flournoy and Frey (1986) (data not shown). Again, a thioester would be cleaved by exposure to pH 12.5, but an acylamide would not be cleaved. Incubation of the ^{14}C-labeled PDH complex at pH 2.5 for 70 min fails to cleave the ^{14}C label from the protein.

The cleavage of the ^{14}C acetyl group from the ^{14}C-labeled enzyme by base and NH_2OH, as well as its stability at pH 2.5, suggested that a cysteinyl sulfhydryl group is acetylated in the inactivated enzyme. If so, this thioester should be reduced by $NaBH_4$ to ethanol. To test this, a sample of [^{14}C]PDH complex was reduced with $NaBH_4$ and combined with carrier ethanol, and the carrier was isolated by distillation. The carrier contained 41% of the radioactivity, and its specific radioactivity remained constant through five distillations (see Experimental Procedures).

If the ^{14}C label in the PDH complex and E_1 component is a [^{14}C]acetyl thioester, the labeled enzyme must have lost a cysteinyl sulfhydryl group that is present in the native enzyme. To examine this, we analyzed both the active E_1 component and the fluoropyruvate-inactivated enzyme for sulfhydryl groups using DTNB as the group-selective test reagent. The active E_1 component was found to contain 6.20 ± 0.02 (SEM) free sulfhydryl groups per molecule, whereas the inactivated enzyme contained 4.83 ± 0.03 free sulfhydryl groups by this assay (Figure 4). Therefore, 1.37 ± 0.03 sulfhydryl groups per molecule was lost in the inactivation reaction, presumably in an acetyl group transfer reaction with acetyl-TPP. This blocked the sulfhydryl group as an acetyl thioester and inactivated the enzyme.

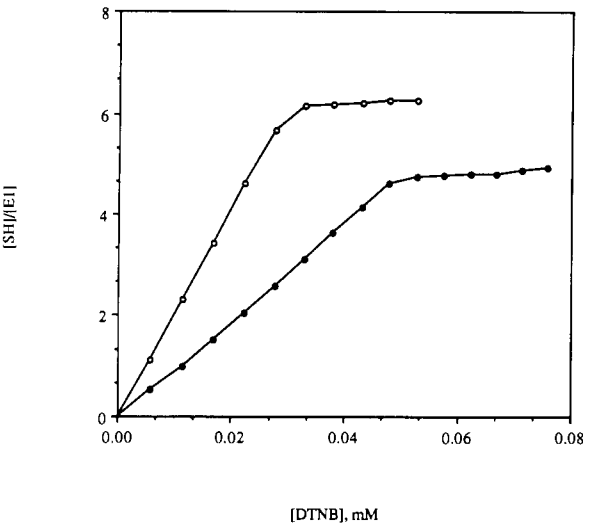


FIGURE 4: Titration of sulfhydryl groups on E_1 using DTNB. The E_1 component in active form (○) and fluoropyruvate-inactivated form (●) was incubated with KP_i buffer (39 mM, pH 7.0) containing urea (8.1 M). Each solution was then titrated with 10-μL aliquots of 0.58 mM DTNB. The absorbance at 412 nm resulting from each addition of DTNB was measured and plotted as a function of total DTNB concentration.

Protection by Dihydrolipoamide against Inactivation by Fluoropyruvate. We have previously reported (Flournoy & Frey, 1986) that acetyl-TPP, generated at the active site of the E_1 component by reaction of fluoropyruvate according to Scheme I, is chemically competent in the acetylation of dihydrolipoamide at high concentrations of this substrate. That is, dihydrolipoamide blocks acetyl group transfer to water through competition. It should, therefore, also compete with a cysteinyl sulfhydryl group at the active site and protect the enzyme against inactivation if pathway B in Scheme II is operative. We examined this question in an experiment in which we assessed the effect of dihydrolipoamide on the rate at which fluoropryvate inactivates the E_1 component. The reaction mixtures consisted of E_1 (1 mg/mL), TPP (1 mM), $MgSO_4$ (1 mM), fluoropyruvate (0.1 mM), and sodium bicinate buffer (34 mM, pH 8.0) in the presence or absence of dihydrolipoamide (25 mM). The dihydrolipoamide was dissolved in ethanol prior to its addition to the reaction mixtures, and the final mixtures contained 10% ethanol by volume. A control reaction mixture contained all the components except fluoropyruvate. The inactivation half-times, measured as described in Figure 2 and under Experimental Procedures, were 4 min in the absence of dihydrolipoamide and 28 min in the presence of dihydrolipoamide. Inhibition of inactivation presumably results from competition between dihydrolipoamide and the enzymic sulfhydryl group for the acetyl group of acetyl-TPP.

Discussion

Fluoropyruvate as an Inactivator of Pyruvate Dehydrogenase (E_1). The results presented in this paper show that the PDH complex and the pyruvate dehydrogenase (E_1) component are both inactivated by fluoropyruvate. The inactivation of both proteins is dependent on the presence of TPP

and is accompanied by covalent modification of E_1, with incorporation of ^{14}C from [^{14}C]fluoropyruvate into the protein. The ^{14}C label is incorporated into the E_1 component of the PDH complex as an acetyl group bonded to a good leaving group. Analysis for sulfhydryl groups indicates that the inactivation results in the loss of at least one sulfhydryl group per molecule in the inactivated enzyme. This is presumably a cysteine residue which is bonded to the acetyl group through sulfur.

Our results appear to conflict with the paper of Bisswanger (1981), who reported that fluoropyruvate does not inactivate the PDH complex. However, Bisswanger's experiments were unlike ours in that he excluded TPP from his reaction mixtures. In one experiment in which TPP was present and at a low level of fluoropyruvate, pyruvate was also present at a high concentration; but we know now that pyruvate protects the enzyme against inactivation by fluoropyruvate. We find that TPP is required both for the E_1-catalyzed reaction of fluoropyruvate to form CO_2, F^-, and acetate and for the suicide inactivation described in this paper. We regard TPP dependence as the most powerful evidence that both of these reactions take place at the active site of E_1.

The kinetics of inactivation by fluoropyruvate are shown in Table I to be complex, with the inactivation rate inhibited by high concentrations of fluoropyruvate. The molecular basis for this inhibition is not known, but it is presumably analogous to substrate inhibition of an enzymatic reaction, in which the reaction is inhibited by the binding of a second molecule of substrate. The analogy may be very close since, as discussed in the following section, the inactivation by fluoropyruvate is a suicide process touched off by the decarboxylase action of the enzyme. Pyruvate dehydrogenase, the E_1 component, is known to catalyze a carboligase activity at high concentrations of the substrate pyruvate to form CO_2 and acetolactate according to eq 3 (Kubasik et al., 1972).

$$2CH_3COCO_2^- + H^+ \longrightarrow CO_2 + CH_3\underset{\displaystyle CH_3}{\overset{\displaystyle OH}{C}}CO_2^- \qquad (3)$$

This reaction occurs in the presence of E_1, pyruvate, TPP, and Mg^{2+} and in the absence of CoASH and NAD^+ or other electron acceptors. These are the conditions under which the reactions of fluoropyruvate are conducted. If fluoropyruvate were to undergo a similar reaction, the inactivation of E_1 would be prevented by the carboligase process. Carboligation would proceed by reaction of 2-fluoro-1-hydroxyethylidene-TPP in Scheme II with the carbonyl group of a second molecule of fluoropyruvate to form fluoroacetofluorolactate prior to the elimination of fluoride. This would prevent the generation of acetyl-TPP in the active site, so that acetylation of the active site sulfhydryl could not take place. Such a process would account for inhibition of inactivation at high concentrations of fluoropyruvate. However, this aspect of the action of fluoropyruvate has not been investigated, and no definite conclusions can be drawn regarding the kinetic complexities in Table I.

Mechanism of Suicide Inactivation by Fluoropyruvate. We have considered two mechanisms in Scheme II to account for the inactivation of the PDH complex by fluoropyruvate. Pathway A has also been suggested by Lowe and Perham (1984) as one of the pathways leading to inactivation of the PDH complex when bromopyruvate is the inactivator, and acetyl-TPP has been suggested as a species leading to inactivation of the mammalian system (Sümegi & Alkonyi, 1983). Neither of these papers firmly established the existence of either mechanism. The evidence presented in this paper lends strong support to mechanism B as the one leading to inactivation of the PDH complex by fluoropyruvate.

We conclude that fluoropyruvate is a suicide inactivator of the pyruvate dehydrogenase component (E_1) in the PDH complex. Fluoropyruvate is processed as an analogue of pyruvate through the decarboxylation step in Schemes I and II. At this point its reaction diverges from that of pyruvate. Instead of undergoing reductive acylation of the lipoyl groups bonded to dihydrolipoyl transacetylase as a normal substrate, 2-fluoro-1-hydroxyethylidene-TPP undergoes elimination of fluoride ion and is thereby transformed into enolacetyl-TPP (Scheme II). Ketonization of the latter generates acetyl-TPP which can react by either of two routes, hydrolysis to acetate or acetyl group transfer to the cysteinyl sulfhydryl group at the active site. Acetylation of the cysteine inactivates the enzyme.

Other investigations have reported the cysteine content of the E_1 component. The primary structure of E_1, derived from the nucleotide sequence, has also been determined (Stephens et al., 1983). By this analysis E_1 contains six cysteine residues, in agreement with the amino acid analysis of Dennert and Eaker (1970), who found 6.1 cysteine residues per molecule (M_r 90 000). A slightly lower cysteine value, 5.1 residues per molecule (M_r 10^5), was reported by Vogel (1971), and a slightly higher value of 6.9 cysteine residues was observed by Eley et al. (1972) for the enzyme from *E. coli* Crookes strain.

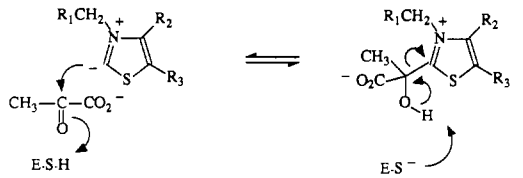
Our cysteine analysis, by titration of the E_1 component under denaturing and nonreducing conditions, indicates the presence of 6.2 cysteine residues, essentially in agreement with other reports. Similar titration of the fluoropyruvate-inactivated enzyme gave 4.8 cysteine residues, showing that inactivation of the pyruvate dehydrogenase subunit is accompanied by the loss of 1.4 cysteine residues per E_1 monomer.

The fact that the cysteine analysis is in agreement with the total cysteine content means that none of the cysteine residues in E_1 are involved in disulfide bonds.

The critical cysteine in our experiments may be the same one whose modification by *p*-mercuribenzenesulfonate inactivates the enzyme (Schwartz & Reed, 1970). These investigators found by titration of the E_1 component with the mercurial in the absence of TPP that two cysteine residues react; one of which is modified without loss of activity and one in which modification inactivates the enzyme. The presence of TPP provided protection from inactivation by mercurials. Lowe and Perham (1984) have observed TPP-dependent modification of a cysteine residue on E_1 by bromopyruvate. In this case, labeling of the PDH complex showed poor specificity and included a TPP-independent component.

Our experiments with fluoropyruvate compared with those of Apfel et al. (1984) with bromopyruvate show that the two suicide inactivators act by different mechanisms to inactivate the PDH complex. Both inactivations are TPP dependent and inhibited by pyruvate, but bromopyruvate reductively bromoacetylates the lipoyl moieties on E_2, and *S*-(bromoacetyl)dihydrolipoyl moieties so formed undergo self destruction by alkylation of their own sulfhydryl groups. Fluoropyruvate does not react by reductive fluoroacetylation of lipoyl moieties but rather eliminates fluoride and generates acetyl-TPP, which then acetylates a sulfhydryl group on E_1. This difference in reactivity suggests that fluoropyruvate may be relatively unreactive in reductive transacetylation. Unreactivity in this case may be related to the high electronegativity of fluorine, which could inhibit electron transfer in reductive transacetylation.

Scheme III



A Possible Role for Cysteine as a Catalytic Group. The present work implicates a cysteine residue at the E_1 active site interacting with a TPP derivative, presumably acetyl-TPP. We cannot be certain that this cysteine plays a chemical role in catalyzing the reaction of pyruvate. But acetylation of this group inactivates the enzyme, either because the sulfhydryl group is directly involved in catalyzing the reaction or because the presence of the acetyl group in the inactivated enzyme alters the structure of the active site and interferes with the normal catalytic process. The acetyl group may simply sterically block the site. In any case it seems certain that the sulfhydryl group is in the active site, since it is acetylated by acetyl-TPP.

The cysteine sulfhydryl group might possibly be a chemical catalyst of the reaction of pyruvate in the active site. The initial reaction of pyruvate with the thiazolium C(2) anion of TPP to form lactyl-TPP requires general-acid catalysis. The cysteinyl SH group may provide this catalysis. And the eventual decomposition of the TPP adduct will require general-base catalysis to remove the proton from the hydroxyl group, and this function might be provided by the cysteinyl S^- (thiolate) group as shown in Scheme III.

Protection by Dihydrolipoamide against Inactivation. We have recently shown (Flournoy & Frey, 1986) that acetyl-TPP formed at the active site of the E_1 component is chemically competent in the acetylation of dihydrolipoamide. This observation led us to consider whether the reductive acetylation of the lipoamide residues on the E_2 subunit of the complex by hydroxyethylidene-TPP may proceed by a mechanism that involves the formation of acetyl-TPP as an intermediate. Such a mechanism might follow eq 4 and 5, which indicate the roles

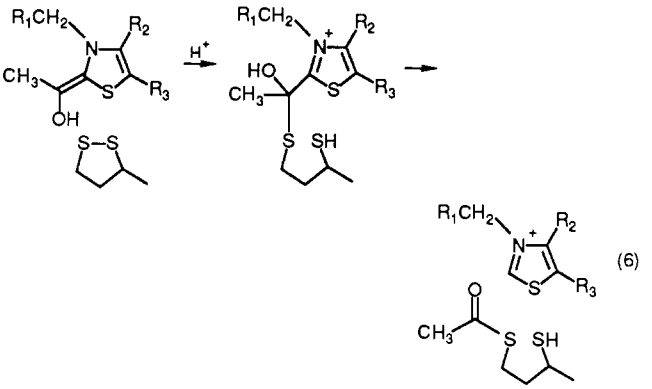
$$\text{hydroxyethylidene-TPP} + \text{LipS}_2 \rightarrow \text{acetyl-TPP} + \text{Lip(SH)}_2 \quad (4)$$

$$\text{acetyl-TPP} + \text{Lip(SH)}_2 \rightarrow \text{TPP} + \text{Lip(SH)-S-acetyl} \quad (5)$$

of the coenzymes TPP and lipoamide [abbreviated LipS_2 and Lip(SH)_2 for the oxidized and reduced forms] but do not explicitly show the essential roles of E_1 and E_2. The present results imply, however, that acetyl-TPP generated from fluoropyruvate at the active site of E_1 inactivates the E_1 component of the complex, raising the question of the credibility of acetyl-TPP as an intermediate in the reaction of pyruvate. Pyruvate is not known to be a suicide substrate under the normal conditions of its reaction catalyzed by the PDH complex in the presence of CoASH and NAD^+.

The hypothetical formation of acetyl-TPP from pyruvate according to eq 4 and 5 is not strictly analogous to the formation of acetyl-TPP from fluoropyruvate at the active site of E_1 in the PDH complex. In the reaction of fluoropyruvate, the lipoamide is *never* reduced. Therefore, acetyl-TPP from fluoropyruvate can only react with water or the sulfhydryl group in the active site of E_1. Acetyl-TPP hypothetically formed from pyruvate would be generated in a reaction that leads by a redox process to the concomitant formation of dihydrolipoamide on E_2 (eq 4). This makes possible a third route by which acetyl-TPP can react further, acetyl group transfer to dihydrolipoamide (eq 5). The dominance of this third route is demonstrated by our earlier work (Flournoy & Frey, 1986) and by our observation in this work that dihydrolipoamide inhibits suicide inactivation by fluoropyruvate, presumably by allowing eq 5 to take place instead of acetyl group transfer to the active site cysteine.

Finally, notwithstanding the fact that acetyl-TPP is chemically competent to be an intermediate and does not inactivate the enzyme when dihydrolipoamide is present at the active site of E_1, there is little direct evidence that it is an intermediate in the reaction of pyruvate. It is detectable at low levels in acid-quenched aliquots of reaction mixtures taken during the PDH complex catalyzed reaction of pyruvate under steady-state conditions.[2] However, there is no compelling evidence that it is a compulsory intermediate. And a mechanism that does not involve the discrete electron transfer and acetyl group transfer steps of eq 4 and 5 can reasonably be written as eq 6. Further research will be required to determine whether eq 4 and 5 or eq 6 correctly presents the mechanism of reductive acetylation of lipoamide by hydroxyethylidene-TPP.



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Alternative Proton Donors/Acceptors in the Catalytic Mechanism of the Glutathione Reductase of *Escherichia coli*: The Role of Histidine-439 and Tyrosine-99†

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ABSTRACT: The cloned *Escherichia coli gor* gene encoding the flavoprotein glutathione reductase was placed under the control of the *tac* promoter in the plasmid pKK223-3, allowing expression of glutathione reductase at levels approximately 40 000 times those of untransformed cells. This greatly facilitated purification of the enzyme. By directed mutagenesis of the *gor* gene, His-439 was changed to glutamine (H439Q) and alanine (H439A). The tyrosine residue at position 99 was changed to phenylalanine (Y99F), and in another experiment, the H439Q and Y99F mutations were united to form the double mutant Y99FH439Q. His-439 is thought to act in the catalytic mechanism as a proton donor/acceptor in the glutathione-binding pocket. The H439Q and H439A mutants retain ~1% and ~0.3%, respectively, of the catalytic activity of the wild-type enzyme. This reinforces our previous finding [Berry et al. (1989) *Biochemistry 28*, 1264-1269] that direct protonation and deprotonation of the histidine residue are not essential for the reaction to occur. The retention of catalytic activity by the H439A mutant demonstrates further that a side chain capable of hydrogen bonding to a water molecule, which might then act as proton donor, also is not essential at this position. Tyr-99 is a further possible proton donor in the glutathione-binding pocket, but the Y99F mutant was essentially fully active, and the Y99FH439Q double mutant also retained ~1% of the wild-type specific activity. Thus, Tyr-99 is not acting as a surrogate proton donor/acceptor to confer activity on the H439Q mutant, and it is unlikely that the phenolic hydroxyl group plays any role in proton transfer in the wild-type enzyme. We conclude that the imidazole side chain of His-439 probably acts as proton donor/acceptor in the wild-type enzyme, leading to an improvement in the k_{cat} of approximately 100-fold compared with the H439Q and H439A mutants. The mutants may be functioning by recruiting another as yet unidentified protein side chain to act in this capacity, but it could be that the proton is simply acquired from solution, given the somewhat open structure of this part of the active site. The H439Q, H439A, and Y99FH439Q mutants all displayed a much lowered K_m for NADPH compared with the wild-type enzyme, although NADPH is bound in a separate site some distance (~18 Å) from that responsible for binding glutathione. The structural basis for this effect remains to be determined.

Glutathione reductase (EC 1.6.4.2) catalyzes the NADPH-linked reduction of oxidized glutathione:

$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+$$

The product of the reaction, reduced glutathione, ensures that other thiol groups remain reduced in the cell and is particularly important in protecting the cell against oxidative stress and in the biosynthesis of DNA [for a review, see Holmgren (1985)]. The catalytic mechanism of the enzyme has been the subject of intensive investigations using a variety of techniques, chiefly protein chemical and kinetic analysis (Williams, 1976) and X-ray crystallography of the human erythrocyte enzyme (Pai & Schulz, 1983; Karplus & Schulz, 1987; Pai et al., 1988; Karplus et al., 1989). From these studies, a reaction scheme has been proposed (Pai & Schulz, 1983; Wong et al., 1988) in which reducing equivalents are passed from NADPH to oxidized glutathione via the isoalloxazine ring of enzyme-bound FAD and the redox-active

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