Griffin, J. H., Rosenbusch, J. P., Blout, E. R., & Weber, K. K. (1973) *J. Biol. Chem. 248*, 5057–5062.
Hammes, G. G., & Wu, C. W. (1971) *Biochemistry 10*, 1051–1057.
Hervé, G., Moody, M. F., Tauc, P., Vachette, P., & Jones, P. T. (1985) *J. Mol. Biol. 185*, 189–199.
Howlett, G. J., Blackburn, M. N., Compton, J. G., & Schachman, H. K. (1977) *Biochemistry 16*, 5091–5099.
Jacobson, G. R., & Stark, G. R. (1973a) *Enzymes (3rd Ed.) 9*, 225–308.
Jacobson, G. R., & Stark, G. R. (1973b) *J. Biol. Chem. 248*, 8003–8014.
Jacobson, G. R., & Stark, G. R. (1975) *J. Biol. Chem. 250*, 6852–6860.
Jayaram, H. N., & Cooney, D. A. (1979) *Cancer Treat. Rep. 63*, 1095–1108.
Johnson, R. S., & Schachman, H. K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1995–1999.
Kantrowitz, E. R., & Lipscomb, W. N. (1977) *J. Biol. Chem. 252*, 2873–2880.
Kantrowitz, E. R., Jacobsberg, L. B., Landfear, S. M., & Lipscomb, W. N. (1977) *Proc. Natl. Acad. Sci U.S.A. 74*, 111–114.
Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980a) *Trends Biochem. Sci. (Pers. Ed.) 5*, 124–128.
Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980b) *Trends Biochem. Sci. (Pers. Ed.) 6*, 150–153.
Kerbiriou, D., & Hervé, G. (1972) *J. Mol. Biol. 64*, 379–392.
Kerbiriou, D., & Hervé, G. (1973) *J. Mol. Biol. 78*, 687–702.
Kerbiriou, D., Hervé, G., & Griffin, J. (1977) *J. Biol. Chem. 252*, 2881–2890.
Kirschner, M. W., & Schachman, H. K. (1973) *Biochemistry 12*, 2997–3004.
Ladner, J. E., Kitchell, J. P., Honzatko, R. B., Ke, H. M., Wolz, K. W., Kalb, A. J., Ladner, R. C., & Lipscomb, W. N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3125–3128.
Landfear, S. M., Evans, D. R., & Lipscomb, W. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2654–2658.
Moody, M. F., Vachette, P., & Foote, A. M. (1979) *J. Mol. Biol. 133*, 517–532.
Murthy, Y. K. S., Thiemann, J. E., Coronelli, C., & Sensi, P. (1966) *Nature (London) 211*, 1198–1199.
Perbal, B., & Hervé, G. (1972) *J. Mol. Biol. 70*, 511–529.
Tauc, P., Leconte, C., Kerbiriou, D., Thiry, L., & Hervé, G. (1982) *J. Mol. Biol. 155*, 155–168.
Thiry, L., & Hervé, G. (1978) *J. Mol. Biol. 125*, 515–534.
Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83.
Weber, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7098–7102.

Active-Site Modification of Mammalian Pyruvate Dehydrogenase by Pyridoxal 5′-Phosphate†

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ABSTRACT: The pyruvate dehydrogenase multienzyme complex from bovine kidney and heart is inactivated by treatment with pyridoxal 5′-phosphate and sodium cyanide or sodium borohydride. The site of this inhibition is the pyruvate dehydrogenase (E_1) component of the complex. Inactivation of E_1 by the pyridoxal phosphate–cyanide treatment was prevented by thiamin pyrophosphate. Equilibrium binding studies showed that E_1 contains two thiamin pyrophosphate binding sites per molecule ($\alpha_2\beta_2$) and that modification of E_1 increased the dissociation constant (K_d) for thiamin pyrophosphate about 50-fold. Incorporation of approximately 2.4 equiv of ^{14}CN per mole of E_1 tetramer in the presence of pyridoxal phosphate resulted in about a 90% loss of E_1 activity. Radioactivity was incorporated predominately into the E_1 α subunit. Radioactive N^6-pyridoxyllysine was identified in an acid hydrolysate of the E_1–pyridoxal phosphate complex that had been reduced with NaB^3H_4. The data are interpreted to indicate that in the presence of sodium cyanide or sodium borohydride, pyridoxal phosphate reacts with a lysine residue at or near the thiamin pyrophosphate binding site of E_1. This binding site is apparently located on the α subunit.

The mammalian pyruvate dehydrogenase complex is organized about a 60-subunit core, consisting of dihydrolipoamide acetyltransferase (E_2)[1] to which multiple copies of pyruvate dehydrogenase (E_1) and dihydrolipoamide dehydrogenase (E_3) are bound by noncovalent bonds (Reed, 1974). In solution, uncomplexed E_1 is a tetramer ($\alpha_2\beta_2$), and E_3 is a homodimer. E_1, E_2, and E_3 act in sequence according to eq 1–5 with eq 6 being the sum of these equations.

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[1] Abbreviations: PDC, pyruvate dehydrogenase complex; E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; TPP, thiamin pyrophosphate; $LipS_2$ and $Lip(SH)_2$, oxidized and reduced lipoic acid, respectively; NAD^+, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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$$CH_3COCO_2H + [TPP]\cdot E_1 \rightarrow [CH_3C(OH)=TPP]\cdot E_1 + CO_2 \quad (1)$$

$$[CH_3C(OH)=TPP]\cdot E_1 + [LipS_2]-E_2 \rightarrow [CH_3CO-S-Lip-SH]-E_2 + [TPP]\cdot E_1 \quad (2)$$

$$[CH_3CO-S-Lip-SH]\cdot E_2 + CoA\text{-}SH \rightarrow [Lip(SH)_2]-E_2 + CH_3CO-S-CoA \quad (3)$$

$$[Lip(SH)_2]-E_2 + [FAD]\cdot E_3 \rightarrow \text{reduced } [FAD]\cdot E_3 + [LipS_2]-E_2 \quad (4)$$

$$\text{reduced } [FAD]\cdot E_3 + NAD^+ \rightarrow [FAD]\cdot E_3 + NADH + H^+ \quad (5)$$

$$CH_3COCO_2H + CoA\text{-}SH + NAD^+ \rightarrow CH_3CO-SCoA + CO_2 + NADH + H^+ \quad (6)$$

Pyridoxal 5′-phosphate has been used as a reagent to identify essential lysine residues in enzymes (Rippa et al., 1967; Schnackerz & Noltmann, 1971; Whitman & Tabita, 1978; Hazra et al., 1984). Pyridoxal phosphate binds reversibly to lysine, forming a Schiff base. The Schiff base can be stabilized by reduction with borohydride or by treatment with cyanide, which adds to the azomethine linkage to form an aminonitrile (Hansen et al., 1974; Bower & Zalkin, 1982). In this paper, we report that pyridoxal phosphate plus sodium cyanide irreversibly inactivates the mammalian pyruvate dehydrogenase complex, and we present evidence that the site of this inhibition is a lysine residue in or near the thiamin pyrophosphate binding site of E_1.

Experimental Procedures

Materials. The pyruvate dehydrogenase complex was isolated from bovine heart and kidney mitochondria and was separated into its component enzymes as described (Pettit & Reed, 1982; Pettit et al., 1982). Pyridoxal 5′-phosphate, *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), thiamin pyrophosphate, $NaBH_4$, and poly(L-lysine) were obtained from Sigma. Hyamine hydroxide, [1-^{14}C]pyruvate, [*thiazole*-2-^{14}C]thiamin hydrochloride, and $Na^{14}CN$ were obtained from Amersham. NaB^3H_4 was obtained from Dr. Esmond Snell.

Assay of Overall Activity of the Pyruvate Dehydrogenase Complex. The overall activity of the complex (eq 6) was determined by monitoring the initial rate of NADH formation at 340 nm and 30 °C with a Gilford recording spectrophotometer. The assay solution contained 2.5 mM NAD^+, 0.1 mM TPP, 0.13 mM CoA, 1 mM $MgCl_2$, 2 mM pyruvate, 50 mM potassium phosphate (pH 8.0), and 0.32 mM dithiothreitol in a final volume of 1 mL. The pH of the solution was 7.4. The reaction was initiated by the addition of enzyme.

Assay of Pyruvate Dehydrogenase (E_1) Activity. Oxidative decarboxylation of [1-^{14}C]pyruvate with ferricyanide as electron acceptor (eq 7) was carried out essentially as described

$$CH_3COCO_2H + 2Fe(CN)_6^{3-} + H_2O \rightarrow CH_3CO_2H + CO_2 + 2Fe(CN)_6^{4-} + 2H^+ \quad (7)$$

by Kresze (1979). The assay mixture (0.1 mL) contained 100 mM Tricine (pH 7.0), 20 mM $CaCl_2$, 100 mM $K_3Fe(CN)_6$, 0.4 mM [1-^{14}C]pyruvate, and enzyme. The reaction mixture was placed in a 1.5-mL conical vial enclosed in a scintillation vial that was sealed with a rubber cap. The scintillation vial contained a paper rectangle soaked with hyamine hydroxide to trap $^{14}CO_2$. Decarboxylation was initiated by addition of [1-^{14}C]pyruvate. At selected time intervals, the reaction was terminated by injection of 0.2 mL of 3 N HCl. After the conical vials were shaken for 1 h, they were removed, and 10 mL of Amersham aqueous counting scintillant was added to each bottle. Radioactivity was measured on a Beckman Model 230 liquid scintillation counter. Some samples of E_1 were assayed for their ability to reconstitute an active pyruvate dehydrogenase complex in the presence of excess E_2 and E_3 (Roche & Reed, 1972).

Assay of Dihydrolipoamide Acetyltransferase (E_2) and Dihydrolipoamide Dehydrogenase (E_3) Activities. The activity of E_2 was determined by two procedures: (i) an excess of E_1 and E_3 was added to E_2 to reconstitute the pyruvate dehydrogenase complex (Bleile et al., 1981), and the mixture was analyzed for activity in the NAD^+ reduction assay (eq 6); and (ii) the rate of the acetyltransferase-catalyzed reaction between [1-^{14}C]acetyl-CoA and exogenous dihydrolipoamide was measured (Butterworth et al., 1975). Assay of E_3 activity was based on the spectrophotometric determination of the rate of NADH oxidation in the presence of exogenous lipoamide (Reed & Willms, 1966).

Synthesis of [^{14}C]Thiamin Pyrophosphate. [^{14}C]TPP was synthesized from [*thiazole*-2-^{14}C]thiamin hydrochloride and polyphosphoric acid by the method of Matsukawa et al. (1970) and purified by ion-exchange chromatography. The reaction was initiated by the addition of 0.5 mL of a hot solution containing 1.06 g of P_2O_5 in 0.65 mL of 85% H_3PO_4 to a vial containing 0.69 mg (2 μmol; 50 μCi) of labeled thiamin hydrochloride. The reaction was allowed to proceed for 15 min on a heating block at 110 °C. After being cooled, the mixture was neutralized with NaOH and diluted to a final phosphate concentration of 20 mM (≈350 mL). The mixture was applied to a DEAE-Sephadex A-25 column (2.5 × 22 cm). The column was developed with 500 mL of a linear gradient from 20 to 500 mM ammonium formate (pH 6.8–7.0). [^{14}C]TPP eluted at 180–200 mM ammonium formate. Fractions containing [^{14}C]TPP were combined and lyophilized. The concentration of [^{14}C]TPP was determined spectrophotometrically at 267 nm in 20 mM potassium phosphate, pH 7.0 (ϵ = 8550 M^{-1} cm^{-1}) (Wittorf & Gubler, 1970). Product purity was checked by chromatography in 1-propanol/1 M ammonium formate (pH 5.0)/H_2O (65:15:25) on Whatman 3MM paper (Krampitz & Votaw, 1966). Labeled compounds were visualized by autoradiography. Unlabeled standards of thiamin, thiamin monophosphate, and TPP were visualized by UV light after the chromatogram was sprayed with $K_3Fe(CN)_6$ in NaOH (Itokawa & Cooper, 1970). As judged from the chromatograms, [^{14}C]TPP isolated from the DEAE column was free of other labeled compounds. The yield was about 0.5 μmol (25–30%), and the specific radioactivity was 20–25 μCi/nmol.

Binding of [^{14}C]Thiamin Pyrophosphate to Pyruvate Dehydrogenase. Binding of [^{14}C]TPP to E_1 was performed in microdialysis cells by equilibrium dialysis as described by Butler et al. (1977). After 4 h at room temperature, 40-μL samples were withdrawn from both sides of the dialysis membrane for determination of free and total [^{14}C]TPP by liquid scintillation counting. The data were fitted to a Scatchard plot (Scatchard, 1949) with the aid of a linear least-squares program. Analyses for hydrolysis of [^{14}C]TPP by chromatography and for proteolysis of E_1 by gel electrophoresis indicated that neither substance was modified during the course of the experiment.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed on slab gels (15% acrylamide) as described by Laemmli (1970). The gels were stained with 0.25% (w/v) Coomassie brilliant blue R in 45% methanol and 10% acetic acid and destained with the same solvent. When radiolabeled proteins were electrophoresed, the stained gels were treated with ENHANCE (New England Nuclear) to decrease expo-

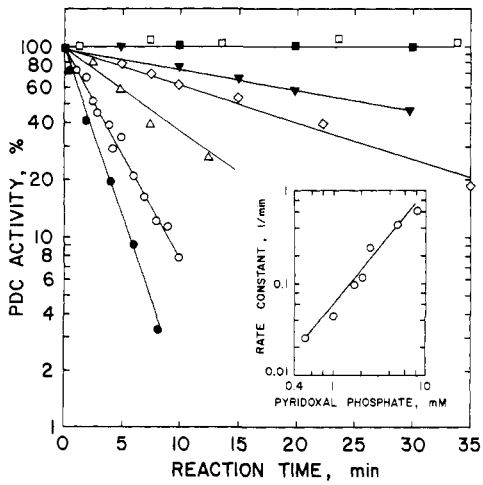

FIGURE 1: Inactivation of pyruvate dehydrogenase complex by pyridoxal phosphate plus NaCN. The reaction mixtures contained 2.5 mg/mL pyruvate dehydrogenase complex, 20 mM potassium phosphate buffer (pH 7.0), 10 mM NaCN, and the following concentrations of pyridoxal phosphate: 0.5 (▼), 1 (◇), 1.75 (△), 2.5 (○), and 5 (●) mM. The final volume was 0.1 mL, and the temperature was 30 °C. At selected time intervals, 5-μL aliquots were taken for assay of overall activity (eq 6). The dilution was 200-fold. The controls lacked either pyridoxal phosphate (□) or pyridoxal phosphate and NaCN (■). Each line represents the average of two to three experiments. Inset: Order of the inactivation process with respect to the concentration of pyridoxal phosphate. The pseudo-first-order rate constants were determined from the slopes of the plots.

sure time during autoradiography at −70 °C. Kodak XAR-5 X-ray film was used. In some experiments, a Dupont Cronex intensifying screen was laid over the film.

Treatment with Pyridoxal Phosphate. Pyruvate dehydrogenase complex and its component enzymes were treated with selected concentrations of pyridoxal phosphate and/or 10 mM NaCN in the dark at 30 °C, or at room temperature for binding experiments, under various conditions described in the text. Some treated samples were dialyzed in the dark at 4 °C for 24 h prior to testing.

Measurement of Protein-Bound Radioactivity. To measure incorporation of radioactivity into protein, samples were spotted onto disks of Whatman 3MM paper. The paper disks were placed immediately into cold 10% trichloroacetic acid. The papers were washed 4 times with 10% trichloroacetic acid, twice with 95% ethanol, and once with diethyl ether. The papers were air-dried, added to scintillation fluid, and counted.

RESULTS

Inactivation of the Pyruvate Dehydrogenase Complex by Pyridoxal Phosphate and NaCN. Preliminary experiments indicated that pyridoxal phosphate inhibited the overall activity (eq 6) of the pyruvate dehydrogenase complex and that the inhibition was reversed by dilution or dialysis. This result probably reflects instability of a lysylpyridoxal phosphate Schiff base. When either sodium borohydride or NaCN was added to stabilize the Schiff base, irreversible inactivation of the pyruvate dehydrogenase complex occurred. Because sodium borohydride, but not sodium cyanide, produced variable inhibition of the pyruvate dehydrogenase complex in the absence of pyridoxal phosphate, subsequent experiments were performed with pyridoxal phosphate plus cyanide. Figure 1 shows the time-dependent inactivation of the pyruvate dehydrogenase complex in the presence of pyridoxal phosphate and 10 mM NaCN. In the absence of pyridoxal phosphate, 10 mM NaCN did not affect the activity. The inactivation followed pseudo-first-order kinetics, and the rate was dependent on pyridoxal phosphate concentration. A plot of the logarithms of the inactivation rate constants vs. the logarithms of the pyridoxal phosphate concentrations was linear (Figure 1, inset). The slope is 1.2, which is the reaction number (Levy et al., 1963), and the second-order rate constant is 78 M^{-1} min^{-1}. The monophasic inactivation kinetics and the reaction number near unity suggest that inactivation resulted from interaction of pyridoxal phosphate with only one type of reactive site on the pyruvate dehydrogenase complex.

Effect of Pyridoxal Phosphate and NaCN on Activities of Component Enzymes of the Pyruvate Dehydrogenase Complex. To further characterize the site of inhibition, the effect of pyridoxal phosphate plus NaCN on the intermediate steps in pyruvate oxidation was determined. E_1, E_2, and E_3 were incubated individually with 2.5 mM pyridoxal phosphate and 10 mM NaCN for 30 min at 30 °C, the mixtures were dialyzed, and aliquots were assayed for their ability to reconstitute an active pyruvate dehydrogenase complex in the presence of E_2 and E_3, E_1 and E_3, and E_1 and E_2, respectively. Treatment of either E_2 or E_3 with pyridoxal phosphate and NaCN resulted in only a 10–15% loss of activity, whereas the same treatment of E_1 resulted in a 78% (±3%, three experiments) loss of its activity. Treatment of a sample of the pyruvate dehydrogenase complex under the same conditions resulted in a 73% (±6%, three experiments) loss of overall activity (eq 6). These observations indicated that E_1 was the site of inhibition by pyridoxal phosphate and NaCN. Additional support for this conclusion is the demonstration that treatment of uncomplexed E_1 with 5 mM pyridoxal phosphate and 10 mM NaCN for 15 min at 30 °C resulted in about 80% inhibition of its ability to catalyze the oxidative decarboxylation of [1-^{14}C]pyruvate with ferricyanide as electron acceptor (eq 7) (data not shown).

Effect of Pyridoxal Phosphate and NaCN on Thiamin Pyrophosphate Binding to Pyruvate Dehydrogenase. Because TPP is the coenzyme for E_1 and because pyridoxal phosphate has been shown to selectively modify primary amino groups in or near phosphate binding sites (Schnackerz & Noltmann, 1971; Whitman & Tabita, 1978), the effect of TPP on the inactivation of E_1 by pyridoxal phosphate and NaCN was examined. Figure 2 shows that TPP plus Mg^{2+}, which is required for TPP binding, prevented the inactivation. $MgCl_2$ alone or pyruvate had no effect (data not shown). The cause of the lag period of 3–5 min in protection by thiamin pyrophosphate plus Mg^{2+} is not known. It is possible that this lag period is sensitive to the order of addition of reagents. Direct evidence that the pyridoxal phosphate–NaCN treatment interfered with TPP binding was obtained by using equilibrium dialysis. Analysis of the data (Figure 3) showed that two TPP binding sites are present per E_1 tetramer ($\alpha_2\beta_2$) with a dissociation constant (K_d) of 6.4 ± 0.5 μM. These values are in good agreement with previous data (Butler et al., 1977). NaCN alone had no effect on K_d. The inhibition was characterized by an increase in K_d for thiamin pyrophosphate to 356 μM at 2 mM pyridoxal phosphate and 10 mM NaCN.

Stoichiometry of Incorporation of ^{14}CN into Pyruvate Dehydrogenase in the Presence of Pyridoxal Phosphate. When uncomplexed E_1 was incubated with Na^{14}CN and pyridoxal phosphate, radioactivity was selectively incorporated into the E_1 α subunit (Figure 4). At 12 min, approximately 0.9 nmol of ^{14}CN was incorporated per nanomole of the E_1 α subunit and about 0.3 nmol per nanomole of the E_1 β subunit. At that time, E_1 was inactivated to about 90%.

In an attempt to identify the amino acid residue to which the pyridoxal phosphate was attached, a sample of E_1 that had been inactivated by treatment with Na^{14}CN and pyridoxal

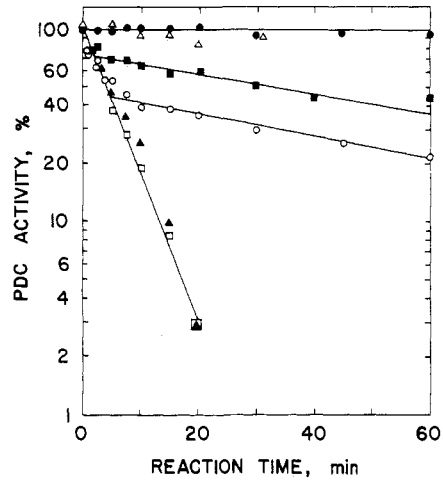

FIGURE 2: Effect of thiamin pyrophosphate on inactivation of pyruvate dehydrogenase complex by pyridoxal phosphate and NaCN. The reaction mixtures contained 2.5 mg/mL pyruvate dehydrogenase complex, 20 mM potassium phosphate (pH 7.0), 10 mM NaCN, 2 mM pyridoxal phosphate (□), and the following substances: 6 mM $MgCl_2$ (▲); 6 mM $MgCl_2$ and 2 mM TPP (○); 10 mM $MgCl_2$ and 10 mM TPP (■). The controls lacked pyridoxal phosphate (△) or pyridoxal phosphate and NaCN (●). Other conditions were as described in the legend of Figure 1.

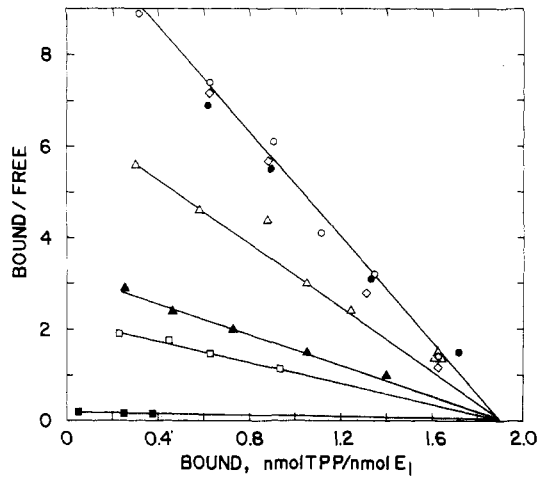

FIGURE 3: Scatchard plots of thiamin pyrophosphate binding to pyruvate dehydrogenase. The upper chamber of the dialysis apparatus contained 0.036 mM E_1 tetramer, 20 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 6 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.013–0.103 mM [^{14}C]TPP, 10 mM NaCN, and the following concentrations of pyridoxal phosphate: 0.1 (△), 0.4 (▲), 0.75 (□), and 2 (■) mM. The controls lacked pyridoxal phosphate (●), NaCN (◇) (0.75 mM pyridoxal phosphate present), or both substances (○). The volume was 80 μL. The lower chamber contained 80 μL of 20 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, and 6 mM $MgCl_2$.

phosphate was dialyzed extensively and then hydrolyzed in 6 N HCl at 110 °C for 24 h. The hydrolysate was subjected to chromatography in 1-butanol/acetic acid/pyridine/H_2O (37.5:7.5:25:30) on a Brinkmann thin-layer cellulose plate. Autoradiographs showed several bands (data not shown). Similar results were obtained with a sample of poly(L-lysine) that had been labeled by incubation with pyridoxal phosphate and Na^{14}CN. However, when a sample of E_1 that had been inactivated by incubation with pyridoxal phosphate and NaB^3H_4 was subjected to acid hydrolysis, only one radioactive band was detected on the chromatogram. This band migrated to the same position as an authentic sample of N^6-pyridoxyllysine prepared by treatment of poly(L-lysine) with pyridoxal phosphate and NaB^3H_4 followed by acid hydrolysis (data not shown).

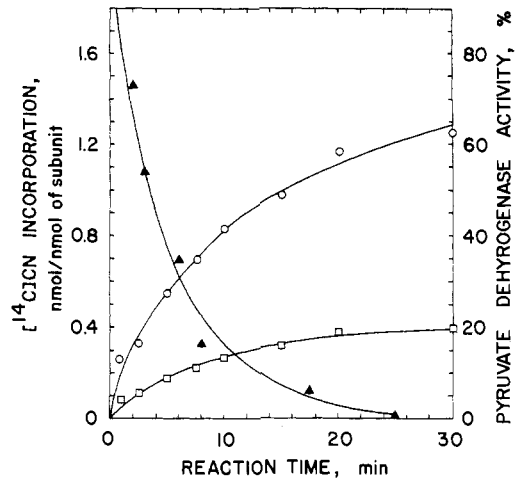

FIGURE 4: Time course of labeling of pyruvate dehydrogenase subunits in the presence of pyridoxal phosphate and Na^{14}CN. The incubation mixture contained 0.75 mg of E_1, 20 mM phosphate buffer (pH 7.0), 2.5 mM pyridoxal phosphate, and 5 mM Na^{14}CN in a final volume of 0.3 mL. At the indicated time intervals, aliquots were taken for determination of enzyme activity (▲) and for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue R. The bands corresponding to the α (○) and β (□) subunits were cut out, dissolved in 30% hydrogen peroxide, and counted. In the absence of pyridoxal phosphate, little radioactivity was found in the α and β bands.

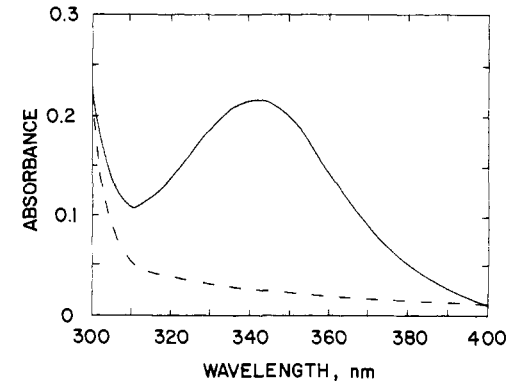

FIGURE 5: Spectra of the native and modified pyruvate dehydrogenase. Spectrum of the native enzyme, 2.5 mg/mL, in 10 mM potassium phosphate, pH 7.0 (---). Spectrum of the enzyme, 2.5 mg/mL, modified with pyridoxal phosphate and NaCN as described under Experimental Procedures (—). Spectra were determined with a Varian Model 2200 recording spectrophotometer.

Absorption Spectra. Pyruvate dehydrogenase that had been treated with pyridoxal phosphate plus NaCN and then dialyzed showed an absorption peak at 340 nm which was not present in the native enzyme (Figure 5). Samples of the enzyme that had been treated with either 5 mM pyridoxal phosphate or 10 mM NaCN alone had absorption spectra similar to the native enzyme. By comparison, the spectrum of anthranilate synthase and poly(L-lysine) modified with pyridoxal phosphate and NaCN showed an absorption peak near 325 nm (Bower & Zalkin, 1982) and at 330 nm, respectively (data not shown).

DISCUSSION

The data reported in this paper demonstrate that the pyruvate dehydrogenase (E_1) component of the mammalian pyruvate dehydrogenase complex is inactivated by chemical modification of a lysine residue in or near the thiamin pyrophosphate binding site. Under the conditions used, the activities of the E_2 and E_3 components of the complex were not significantly affected. Pyridoxal phosphate alone caused

variable inhibition of E_1, and the inhibition was reversed by dilution or dialysis. Activity was lost irreversibly after treatment of E_1 with pyridoxal phosphate and either sodium borohydride or sodium cyanide.

Inactivation of E_1 by the pyridoxal phosphate–cyanide treatment was prevented by thiamin pyrophosphate, but not by pyruvate. Equilibrium binding studies showed that modification of E_1 by pyridoxal phosphate–cyanide treatment decreased the K_d for thiamin pyrophosphate about 50-fold. E_1 binds 2 mol of thiamin pyrophosphate per mole of enzyme ($\alpha_2\beta_2$), as reported previously (Butler et al., 1977) and confirmed in the present investigation.

The stoichiometry of the interaction of E_1 with pyridoxal phosphate and NaCN was determined by using $Na^{14}CN$. Approximately 2.4 equiv of ^{14}CN was incorporated in the presence of pyridoxal phosphate per mole of E_1 tetramer, concomitant with a 90% loss of E_1 activity. ^{14}CN was incorporated predominately into the α subunit. We interpret these results to indicate that the TPP binding site of E_1 is located on the α subunit. It is interesting to note that a kinase-catalyzed phosphorylation and inactivation of E_1 occurs on a unique serine residue in the α subunit (Barrera et al., 1972; Yeaman et al., 1978). Also, binding of TPP to E_1, presumably at the active site, inhibits the kinase activity, apparently by altering the conformation about the phosphorylation site on the α subunit so that the serine hydroxyl group is less accessible to the kinase (Butler et al., 1977). It has been proposed that the α subunit catalyzes reaction 1 and that the β subunit catalyzes reaction 2 (Roche & Reed, 1972; Hübner et al., 1978). However, attempts thus far to separate the α and β subunits with retention of catalytic activity have been unsuccessful.

Acknowledgments

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Registry No. E_1, 9001-04-1; PDC, 9014-20-4; NaCN, 143-33-9; $NaBH_4$, 16940-66-2; pyridoxal 5′-phosphate, 54-47-7; thiamin pyrophosphate, 154-87-0; L-lysine, 56-87-1.

References

Barrera, C. R., Namihira, G., Hamilton, L., Munk, P., Eley, M. H., Linn, T. C., & Reed, L. J. (1972) *Arch. Biochem. Biophys. 148*, 343–358.

Bleile, D. M., Hackert, M. L., Pettit, F. H., & Reed, L. J. (1981) *J. Biol. Chem. 256*, 514–519.

Bower, S., & Zalkin, H. (1982) *Arch. Biochem. Biophys. 219*, 121–127.

Butler, J. R., Pettit, F. H., Davis, P. F., & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun. 74*, 1667–1674.

Butterworth, P. J., Tsai, C. S., Eley, M. H., Roche, T. E., & Reed, L. J. (1975) *J. Biol. Chem. 250*, 1921–1925.

Hansen, B. A., Lane, R. S., & Dekker, E. E. (1974) *J. Biol. Chem. 249*, 4891–4896.

Hazra, A. K., Detera-Wadleigh, S., & Wilson, S. H. (1984) *Biochemistry 23*, 2073–2078.

Hübner, G., Neef, H., Schellenberger, A., Bernhardt, R., & Khailova, L. S. (1978) *FEBS Lett. 86*, 6–8.

Itokawa, Y., & Cooper, J. R. (1970) *Methods Enzymol. 18A*, 91–92.

Krampitz, L. O., & Votaw, R. (1966) *Methods Enzymol. 9*, 65–70.

Kresze, G.-B. (1979) *Anal. Biochem. 98*, 85–88.

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

Levy, H. M., Leber, P. D., & Ryan, E. M. (1963) *J. Biol. Chem. 238*, 3654–3659.

Matsukawa, T., Hirano, H., & Yurugi, S. (1970) *Methods Enzymol. 18A*, 141–162.

Pettit, F. H., & Reed, L. J. (1982) *Methods Enzymol. 89*, 376–386.

Pettit, F. H., Yeaman, S. J., & Reed, L. J. (1982) *Methods Enzymol. 90*, 195–200.

Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–46.

Reed, L. J., & Willms, C. R. (1966) *Methods Enzymol. 9*, 247–265.

Rippa, M., Spanio, L., & Pontremoli, S. (1967) *Arch. Biochem. Biophys. 118*, 48–57.

Roche, T. E., & Reed, L. J. (1972) *Biochem. Biophys. Res. Commun. 48*, 840–846.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Schnackerz, K. D., & Noltmann, E. A. (1971) *Biochemistry 10*, 4837–4843.

Whitman, W. B., & Tabita, F. R. (1978) *Biochemistry 17*, 1282–1287.

Wittorf, J. H., & Gubler, C. J. (1970) *Eur. J. Biochem. 14*, 53–60.

Yeaman, S. J., Hutcheson, E. T., Roche, T. E., Pettit, F. H., Brown, J. R., Reed, L. J., Watson, D. C., & Dixon, G. H. (1978) *Biochemistry 17*, 2364–2370.