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Fluorescence Energy Transfer Measurements in the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with Chemically Modified Lipoic Acid†

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ABSTRACT: The lipoic acid residues of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* have been modified with radioactive *N*-ethylmaleimide, *N*-(3-pyrene)maleimide, and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM). The number of moles of label incorporated per 4.6×10^6 molecular weight is 47 with *N*-ethylmaleimide, 48 with *N*-(3-pyrene)maleimide, and 40 with DDPM. The last two numbers are less precise than the first because of uncertainties in the extinction coefficients of the enzyme bound labels. The overall activity of the enzyme is abolished by modification of the lipoic acid, but the enzymatic activities of pyruvate dehydrogenase and dihydrolipoyl dehydrogenase are unaltered. Direct binding measurements with 1,N^6-etheno-CoA indicate that the number of CoA specific sites of the dihydrolipoyl transacetylase is unaltered when the lipoic acid is modified with DDPM, but the dissociation constant increases about a factor of four to 218 μM (0.02 M potassium phosphate, pH 7.0, 5 °C). This suggests that lipoic acid interacts with the catalytic site of the transacetylase enzyme. Fluorescence lifetimes were used to measure fluorescence energy transfer within the enzyme complex using the following energy donors: thiochrome diphosphate, bound to the catalytic site of the pyruvate dehydrogenase enzyme; 8-anilino-1-naphthalenesulfonate, bound to the acetyl-CoA regulatory site on the pyruvate dehydrogenase enzyme; or *N*-(3-pyrene)-maleimide, bound to the lipoic acid on the dihydrolipoyl transacetylase enzyme. The energy acceptors were DDPM, bound to the lipoic acid, or FAD, bound to the dihydrolipoyl dehydrogenase enzyme. No energy transfer was observed between the modified lipoic acid groups and the labeled sites on the pyruvate dehydrogenase or the dihydrolipoyl dehydrogenase. Therefore, the distance between the lipoic acid and these sites must be greater than 40 Å assuming the emission and absorption dipoles are randomly oriented. These results and others previously reported suggest that the simple mechanism of a single lipoic acid rotating between the catalytic sites of the three enzymes is unlikely. An alternative mechanism consistent with existing data is that two or more lipoic acids are used to transfer the intermediates between the three catalytic sites of functionally coupled enzymes in a single catalytic cycle.

The purified pyruvate dehydrogenase multienzyme complex from *E. coli* has been shown to contain three component enzymes which catalyze the overall reaction (Koike et al., 1960)

$$CH_3COCO_2H + CoA + DPN^+ \rightarrow CO_2 + CH_3CO\text{-}CoA + DPNH + H^+ \quad (1)$$

The pyruvate dehydrogenase (E_1)[1] contains regulatory binding sites for acetyl-CoA and GTP plus the catalytic binding sites for pyruvate and the cofactor thiamin diphosphate. The dihydrolipoyl transacetylase (E_2) contains the binding sites for CoA and lipoic acid covalently attached through an ϵ-amino group of lysine. The dihydrolipoyl dehydrogenase (E_3) contains FAD at the active site. A model has been proposed, based on structural and chemical evidence, in which 24 polypeptide chains of E_1, 24 polypeptide chains of E_2, and 12 polypeptide chains of E_3 are arranged such that the catalytic sites for each local grouping of the three enzymes are within a sphere of radius 14 Å with the radius being defined by the lipoic acid–lysine prosthetic group. The lipoic acid group is postulated to transfer the hydroxyethyl intermediate from the active site of E_1 via a reductive acetylation of lipoic acid to the active site of E_2 where acetyl CoA is formed. The reduced lipoic acid is then reoxidized by the FAD of E_3 with the substrate DPN^+ oxidizing the FAD. A modification of this mechanism allows disulfide interchange of the intermediates between two lipoic acids which doubles the lipoic acid requirement and expands the diameter of the sphere containing the three catalytic sites to 56 Å (Koike et al., 1963).

Previous work with this multienzyme complex (Moe et al., 1974; Shepherd and Hammes, 1976; Shepherd et al., 1976; Papadakis and Hammes, 1977) has been concerned with measurements of intersubunit and intrasubunit distances between specific ligand binding sites or specific labels attached by covalent modification. The results obtained imply that the E_1, E_2, and E_3 subunits are arranged with their catalytic sites farther apart than the distances required for the single rotating lipoic acid mechanism although the intersubunit distances are

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; ϵCoA, 1,N^6-etheno-coenzyme A; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DPN^+, nicotinamide adenine dinucleotide; DPNH, reduced DPN; E_1, pyruvate dehydrogenase; E_2, dihydrolipoyl transacetylase; E_3, dihydrolipoyl dehydrogenase; FAD, flavin adenine dinucleotide; NEM, *N*-ethylmaleimide; NPR, *N*-(3-pyrene)maleimide; $NaDodSO_4$, sodium dodecyl sulfate; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

still compatible with the multiple lipoic acid model.

The work presented here continues these specific site-to-site fluorescence energy-transfer measurements. Substituted maleimide derivatives of the lipoic acids of E_2 in the intact multienzyme complex were prepared. These covalent and nondissociable labels were the chromophore DDPM and the fluorophore NPR. The results indicate that the derivatized lipoic acid groups are at least 40 Å from the catalytic site of E_1, the acetyl CoA binding site of E_1, and the catalytic site of E_3. The binding of ϵCoA to the active site of E_2 is considerably weaker when the lipoic acid is modified with DDPM. The number of lipoic acids modified in the multienzyme complex was determined with radioactive NEM and spectroscopically with DDPM and NPR. The number of modified lipoic acids found was 47 for NEM, 40 for DDPM, and 48 for NPR.

Materials and Methods

Chemicals. The ϵCoA was obtained from P-L Biochemicals as the lithium salt, the quinine bisulfate and DDPM were from Aldrich Chemical Co., NPR was from Regis, the [^{3}H]NEM was from New England Nuclear, and all other biochemicals were from Sigma. The purity and preparation of the ϵCoA solutions were as described before (Shepherd et al., 1976). All other chemicals were the best available commercial grades, and deionized distilled water was used in all solutions.

Pyruvate Dehydrogenase Complex and Maleimide Derivatives. The preparation and purification of the enzyme complex from *E. coli,* Strain B (Miles Labs) were as previously described (Reed and Willms, 1966). The overall activity of the complex was determined using the DPN reduction assay (eq 1) at 30 °C; the activity of the E_3 component was determined by the lipoamide reduction assay (Reed and Willms, 1966); and the activity of the E_1 component was determined by the ferricyanide reduction assay (Schwartz et al., 1968). The apo complex, with greater than 99% of the thiamin diphosphate removed, was prepared as previously described (Shepherd and Hammes, 1976).

The enzyme complex was labeled with the maleimides in the presence of thiamin diphosphate and pyruvate in order to specifically modify the lipoic acid (Grande et al., 1975). In order to minimize nonlipoate labeling, the enzyme complex was first reacted with nonradioactive NEM. The reaction mixture contained 4.8 mM NEM 4 μM apo-enzyme complex in 0.02 M potassium phosphate (pH 7.0) at 4 °C. At 30-min intervals, the reaction mixture was assayed using the DPN reduction assay for the overall enzyme complex activity. After 4 h, the reaction was quenched with an aliquot of dithiothreitol sufficient to scavenge the excess maleimide. The reaction mixture was passed through a G-25 Sephadex (Pharmacia) column (25 cm × 1.75 cm i.d.) equilibrated with the reaction medium buffer at 4 °C. The void volume eluent containing the enzyme complex was collected by monitoring the fluorescence (360-nm excitation) of the FAD (520-nm emission). The enzyme was precipitated with 45% ammonium sulfate, and the precipitate obtained after centrifugation was suspended in 1 mL of the reaction medium buffer. This solution was dialyzed for 24 h against two changes of 1000 volumes of the same buffer. The final solution was clarified by centrifugation at 12 000*g* for 20 min.

This preblocked enzyme complex had lost no E_1, E_3, or overall enzyme activity and the lipoic acid residues of this enzyme were modified with various maleimide derivatives. The reaction mixture in 0.02 M potassium phosphate (pH 7.0) at 4 °C contained 0.5 mM thiamin diphosphate, 4.3 mM pyruvate, 2.2 mM $MgCl_2$, 4.2 μM DDPM, 325 μM [^{3}H]NEM, or 360 μM NPR. The reaction was initiated by the addition of the maleimide component, and the total reaction volume was 1 mL. At 5-min intervals, the reaction mixture was assayed using the DPN reduction assay for the overall enzyme complex activity. When the activity was less than 10% of the unreacted enzyme complex, the reaction was quenched with an aliquot of dithiothreitol sufficient to scavenge the excess maleimide. The labeled enzyme was then purified and concentrated as described for the preblocking reaction. The protein concentration was determined by the method of Lowry (Lowry et al., 1951).

For some of the energy transfer measurements, the FAD was reduced in the E_3 enzyme component using sodium hyposulfite. The extent of reduction at 4 °C in 0.02 M potassium phosphate (pH 7.0) was monitored by observing the decrease in fluorescence at 520 nm (450-nm excitation). A 100:1 molar ratio of hyposulfite to FAD was sufficient to reduce the FAD under anaerobic conditions. The use of a higher molar ratio of hyposulfite caused precipitation of the protein.

Binding Measurements. The binding of ϵCoA to the DDPM derivatized enzyme complex was determined by the technique of forced dialysis at 4 °C (Cantley and Hammes, 1975). The procedures and methods for determining the free and bound ligand concentrations were as previously described (Shepherd et al., 1976).

The binding of thiochrome diphosphate to the DDPM-labeled enzyme complex was studied by determining the steady-state kinetic inhibition constant of the ferricyanide reduction assay for E_1 at 30 °C in 0.05 M Tricine (pH 7.5) and by fluorescence polarization measurements in 0.02 M potassium phosphate (pH 7.0) at 5 °C (Moe and Hammes, 1974). The excitation wavelength was 365 nm and the emission wavelength was 440 nm for the polarization measurements.

The binding of ANS to the DDPM- and NPR-labeled enzyme complex was studied by fluorescence titrations and the method of continuous variation in 0.02 M potassium phosphate (pH 7.0) at 4 °C (Shepherd and Hammes, 1976).

Spectroscopic Measurements. Ultraviolet and visible absorption measurements were made with a Zeiss PMQII or a Cary 118C spectrophotometer. Steady-state fluorescence measurements and fluorescence polarization measurements were made with a Hitachi Perkin-Elmer MPF-3 fluorescence spectrophotometer. For fluorescence measurements, the cells were thermostated at the desired temperature, and dry nitrogen was circulated through the sample chamber. Corrected emission spectra were determined by comparison with the emission spectrum of quinine bisulfate in 0.1 N H_2SO_4 with excitation at 367 nm or 350 nm at 23 °C (Melhuish, 1962; Chen, 1967). The absolute quantum yield of quinine bisulfate was taken as 0.70 (Scott et al., 1970). Corrections were made for the excitation wavelength shift relative to 313 nm, the inner filter effects associated with absorption of exciting light and of emitted fluorescence, and the anisotropy of the fluorescent species (Shinitzky, 1972).

Correction for the unequal transmission of the horizontal and vertical components of polarized light was made in steady-state polarization measurements (Azumi and McGlynn, 1962). Measurements of the limiting polarization, P_0, for the NPR–mercaptoethanol adduct at 5 °C were made using glycerol to vary the viscosity of 0.02 M potassium phosphate (pH 7.0). The value of P_0 was determined by extrapolation of plots of $1/P - 1/3$ vs. T/η (Perrin, 1926) to $T/\eta = 0$ using eq 2

$$(1/P - 1/3) = (1/P_0 - 1/3)(1 + RT\tau/\eta V) \qquad (2)$$

where R is the gas constant, T is the absolute temperature, τ is the excited state lifetime, V is the molar volume, and η is the

viscosity of the solvent. The values of η at 5 °C for glycerol-buffer solutions were interpolated from graphs of the viscosity as a function of glycerol weight percent and of temperature (Hodgman, 1963).

Fluorescence Lifetime Measurements. An ORTEC 9200 single photon nanosecond fluorescence system was used for fluorescence lifetime measurements as described previously (Matsumoto and Hammes, 1975). Samples were thermostated at 4 °C and dry N_2 was circulated through the sample chamber. An interference filter (Ditric) with maximum transmittance at 435.8 nm (7.4-nm bandpass), 470 nm (6.5-nm bandpass), or 520 nm (8.2-nm bandpass) was used for the emitted light. The excitation wavelength was fixed by an interference filter with maximum transmittance at 340 nm (13.5-nm bandpass), 350 nm (10.3-nm bandpass), 370 nm (10.8-nm bandpass), or 450 nm (6.5-nm bandpass). Light scattering spectra of the enzyme with FAD oxidized or reduced in the absence of ligands were accumulated to be used as reference spectra. The treatment of the data and fitting procedures were as described before (Shepherd and Hammes, 1976).

The anisotropy decay of the bound NPR in the enzyme complex at 5 °C was studied using fluorescence depolarization (Yguerabide, 1972). The sample of 0.82 μM NPR-enzyme complex in 0.02 M potassium phosphate (pH 7.0) was excited at 340 nm and the emission was observed at 380 nm.

Gel Electrophoresis. Polyacrylamide gel electrophoresis with $NaDodSO_4$ was carried out according to the procedure of Weber and Osborn (1969). The 10.0 × 0.5 cm gels contained 7.5% acrylamide and 0.105 *N,N'*-methylenebisacrylamide polymerized in 0.01 M sodium phosphate (pH 7) containing 0.1% $NaDodSO_4$ and 0.1% mercaptoethanol. The electrophoresis in a Gradipore flowcell apparatus (Isolab Inc.) was voltage regulated at 20 V for the first hour, then at 50 V until the tracking dye was within 1 cm of the end of the tube (4–5 h). After removal from the tubes, the gels were observed under 360-nm light, and the positions of fluorescent bands and gel length were determined. Since the amount of protein applied per gel was relatively high (100 μg), the fluorescence due to FAD and the PM was easily detected. Some of the gels were stained in 0.25% Coomassie brilliant blue and destained in 45% methanol, 9% acetic acid. The gels were stored in 5% methanol, 7.5% acetic acid (if stained) or in 45% methanol, 9% acetic acid (if unstained).

The gels containing [^{3}H]NEM-labeled proteins were divided into 1-mm slices using a Mickle gel slicer (Brinkmann Instrument Inc.). The slices were incubated in 1 mL of NCS tissue solubilizer (Amersham/Searle) (9:1, NCS:water) in sealed scintillation vials for 12 h at 50 °C. After cooling, 10 mL of Aquasol-2 scintillation fluid (New England Nuclear) was added to each vial, and the radioactivity was determined in a Beckman LS-255 liquid scintillation counter. Appropriate corrections were made for background and quenching. The overall incorporation of [^{3}H]NEM into the enzyme complex was determined using the protein concentration of the labeled enzyme and the specific activity of [^{3}H]NEM. Aliquots of the labeled enzyme were added to 10 mL of Aquasol-2 scintillation fluid, and the radioactivity was determined as above.

Results

Properties of Maleimide Labeled Enzyme Complexes. In all cases, modification of the enzyme complex with the maleimides as described in the Experimental Section led to the loss of about 95% of the overall activity of the complex. However, the losses in specific activity of the E_1 and E_3 enzymes were less than 5%. If the reaction is carried out in the absence of thiamin diphosphate or pyruvate, the maleimide is incorporated into E_1 without the loss of E_1 or overall enzyme complex activity. The derivatized enzyme complex was much less stable with respect to freezing than the native enzyme. The labeled enzyme solutions lost substantial amounts of FAD and displayed increased turbidity after just one freeze/thaw cycle. The loss of FAD was determined from fluorescence excitation spectra, and the loss of E_3 activity was determined from the lipoamide assay. Polyacrylamide gel electrophoresis in 0.1 M sodium phosphate (pH 7) indicated the enzyme complex was not dissociated by the chemical modifications.

The incorporation of NEM into the multienzyme complex (prelabeled as described in the Experimental Section) was determined using [^{3}H]NEM (specific activity 142 Ci/mol) and was found to be 47 mol of NEM per 4.6 × 10^6 molecular weight protein. The $NaDodSO_4$-polyacrylamide gels indicated less than 5% of the total radioactive label in the band associated with E_3, with the remainder of the radioactivity in the gel band associated with E_2 or in undissociated high molecular weight aggregates. The NEM-labeled enzyme complex retained approximately 6% of its overall activity, and the half-time for the loss of this activity was 6 min at 5 °C.

The stoichiometry of the DDPM reaction with the prelabeled multienzyme complex was found to be 40 mol of DDPM per 4.6 × 10^6 molecular weight protein. This value was determined from the difference spectra of the labeled and unlabeled enzyme complex at 5 °C using an extinction coefficient at 440 nm of 3000 M^{-1} cm^{-1} (Gold and Segal, 1964). The time for this reaction to reach 90% inactivation of the complex was 6 min. Due to the relatively low absorbance and nonfluorescence of this maleimide, the location of DDPM on unstained $NaDodSO_4$-polyacrylamide gels could not be determined directly.

The exact amount of NPR incorporated into the multienzyme complex is difficult to determine. The absorption spectrum of the NPR has a major absorption peak at 340.5 nm in 0.02 M potassium phosphate (pH 7.0); this peak is slightly shifted to the red in nonpolar solvents. In dioxane, the absorption spectrum of the NPR-mercaptoethanol adduct has a peak at 342 with an estimated extinction coefficient of 38 000 M^{-1} cm^{-1} (Holowka and Hammes, 1978). The difference spectrum (vs. unlabeled enzyme) (Figure 1) of the NPR-labeled enzyme complex has an absorption peak at 345 nm suggesting a much more hydrophobic NPR environment and a correspondingly higher extinction coefficient. The absorption spectrum of NPR-labeled enzyme in the $NaDodSO_4$-polyacrylamide gel incubation buffer, corrected for the native enzyme absorption, has an absorption peak at 342.5 nm. The measured absorption in $NaDodSO_4$, the known protein concentration, and the extinction coefficient found in dioxane indicate 48 mol of NPR are bound per 4.6 × 10^6 molecular weight protein. This stoichiometry is only approximate because of the uncertainty in the extinction coefficient. The labeled complex retained 6–8% of the initial activity, and the half-time for the inactivation was 13 min. The $NaDodSO_4$-polyacrylamide gels of the NPR-labeled enzyme complex revealed the blue-white fluorescence of NPR primarily in the region of the gel where the E_2 band is found with faint bands in the region of undissociated higher aggregates.

The steady-state quantum yield of the bound NPR calculated on the basis of the above stoichiometry is 0.133 at 5 °C in 0.02 M potassium phosphate (pH 7.0) with a 350-nm excitation wavelength; the corrected emission spectrum is shown in Figure 1. The polarization of the enzyme-bound NPR (5 °C) was found to be 0.075 with excitation at 344 nm and emission at 450 nm. The dynamic anisotropy determined by nanosecond depolarization measurements gave a rotational correlation time

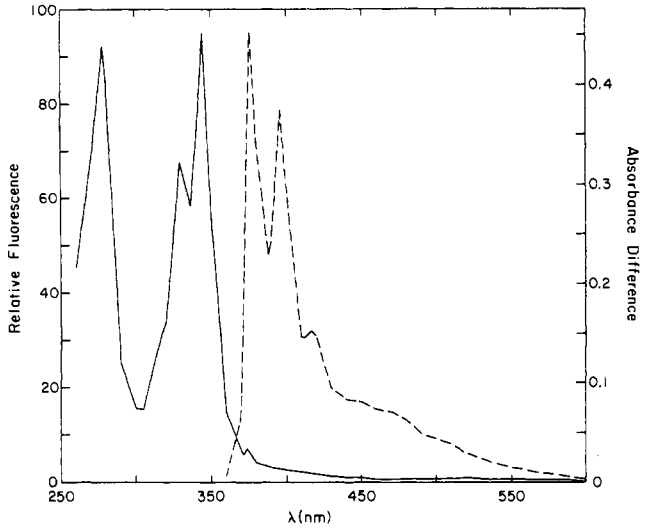

FIGURE 1: A plot of the absorption spectrum and corrected fluorescence emission spectrum for NPR bound to the pyruvate dehydrogenase enzyme complex. The absorption spectrum (—) was obtained from a difference spectrum of the labeled and the unlabeled enzyme complex (0.213 μM) at 5 °C in 0.02 M potassium phosphate (pH 7.0). The corrected fluorescence emission spectrum (350-nm excitation) (- - -) was obtained at 5 °C with 0.112 μM NPR labeled enzyme complex in 0.02 M potassium phosphate (pH 7.0).

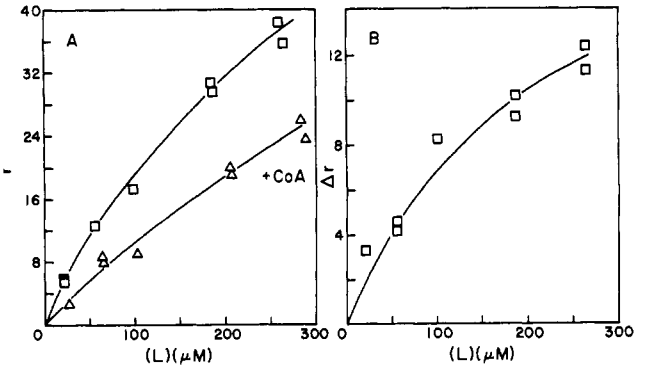

FIGURE 2: (A) A plot of the number of moles of ϵCoA bound per mole of DDPM labeled pyruvate dehydrogenase complex, r, vs. the concentration of free ϵCoA [(L)]. The data were obtained by forced dialysis at 5 °C: (□) 0.54 or 1.98 μM DDPM labeled complex, 32–602 μM ϵCoA; (△) 0.54 or 1.98 μM DDPM labeled complex, 32–602 μM ϵCoA, and 350 or 650 μM CoA. All solutions contained 7.0 mM Mg^{2+}, 1.7 mM dithiothreitol, and 0.02 M potassium phosphate (pH 7.0). The data plotted are the average of duplicate determinations; the lines have no theoretical significance. (B) A plot of the number of moles of ϵCoA bound per mole of DDPM labeled complex that are displaceable by saturating CoA (350 or 650 μM), Δr, vs. the concentration of free ϵCoA found in the absence of CoA [(L)]. The data are from A. The line is a least-squares fit using eq 3 with n = 21.8 and K_D = 218 μM.

of 300–400 ns at 5 °C. The value of the limiting polarization for a NPR–mercaptoethanol adduct with 344-nm excitation and 370-nm emission is 0.255. The polarization of the NPR-labeled complex for the same spectral transition is 0.182. The polarization of the NPR–enzyme complex was not changed by titration of the enzyme with thiamin diphosphate (to 45 μM), pyruvate (to 460 μM), CoA (to 88 μM), or DPN^+ (to 165 μM) or by various combinations of these ligands. The only exception to this finding was that in the presence of high concentrations of all substrates and cofactors used in the DPN^+ reduction assay, a 5% increase in polarization occurred. However, urder these conditions some DPNH is formed which may be influencing the measured polarization.

Binding Measurements. The binding of ϵCoA to DDPM-labeled complex was studied in the presence and absence of saturating CoA at 5 °C. The results obtained are summarized in Figure 2A as a plot of r, the moles of ligand bound per mole of enzyme complex, vs. the free ϵCoA concentration. The measurements were carried out with solutions containing 0.54 or 1.98 μM DDPM-labeled enzyme, 7.0 mM Mg^{2+}, 1.7 mM dithiothreitol, 32–602 μM ϵCoA, and in experiments with saturating CoA, 350 or 650 μM CoA. Although ϵCoA binds to more sites in the complex than are displaceable by CoA, the binding isotherm for the CoA specific sites can be constructed from the data by plotting Δr vs. the free ligand concentration (Figure 2B) where Δr is the difference in r values for ϵCoA binding with and without a saturating concentration of CoA at the same total ligand and enzyme concentrations.

The data in Figure 2B were fit by a nonlinear least-squares analysis to eq 3

$$\Delta r = n(L)/[K_D + (L)] \quad (3)$$

which assumes n independent binding sites characterized by a dissociation constant, K_D, and (L) is the free ligand concentration. The results obtained gave n = 21.8 sites and K_D = 218 μM; the curve in Figure 2B was calculated with these parameters and eq 3.

The steady-state kinetic inhibition constant of thiochrome diphosphate for the DDPM-labeled enzyme in 0.05 M Tricine (pH 7.5) at 30 °C was found to be 12.3 μM. The fluorescence polarization of the thiochrome diphosphate was determined while titrating 1.68 μM DDPM labeled enzyme or unlabeled enzyme with 730 μM thiochrome diphosphate to a final total concentration of 106 μM. The polarization of the bound thiochrome diphosphate was determined by extrapolation of a plot of P, the observed polarization, vs. $(L)/(L_T)$ to $(L)/(L_T) = 0$ according to eq 4

$$P = P_B + [(L)/(L_T)](P_F - P_B) \quad (4)$$

where $(L)/(L_T)$ is the ratio of free ligand to total ligand and P_F is the polarization of free ligand (0.062). In deriving eq 4 it was assumed that the quantum yields of free and bound ligand were equal. The values of (L) were calculated using the parameters determined previously (Moe and Hammes, 1974). The value of P_B was found to be 0.255 for the binding of ligand to the unmodified complex and 0.221 for binding to the DDPM modified complex.

The difference spectrum of thiochrome diphosphate bound to the unlabeled enzyme complex at 5 °C in 0.02 M potassium phosphate (pH 7.0) shows an isosbestic point at 367 nm with a maximum positive change in extinction coefficient at 390 nm and a maximum negative change in extinction coefficient at 335 nm.

The binding of ANS to the DDPM-labeled and NPR-labeled enzyme complex, as determined by fluorescence titration and the method of continuous variation, was essentially the same as with the native enzyme complex. The decrease in ANS fluorescence induced by titration of the enzyme with thiamin diphosphate and pyruvate was also identical with that observed with the unlabeled enzyme complex (Shepherd and Hammes, 1976).

Fluorescence Lifetime Measurements. The fluorescence lifetime experiments were designed to measure fluorescence energy transfer within the pyruvate dehydrogenase enzyme complex between three energy donors, thiochrome diphosphate, NPR, and ANS and the potential energy acceptors FAD and DDPM. The thiochrome diphosphate binds at the catalytic site of E_1 and was used as an energy donor to FAD, bound at the catalytic site of E_3, or to DDPM, bound to a lipoic

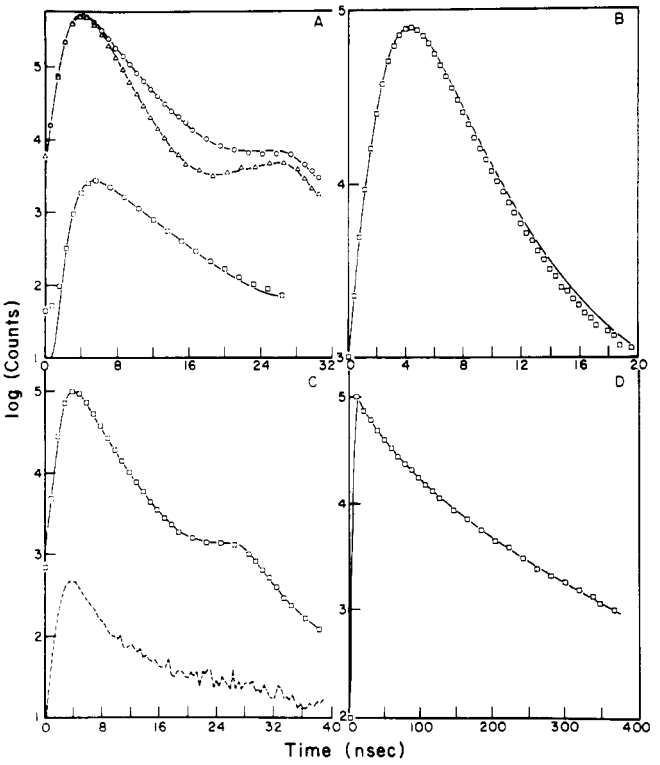

FIGURE 3: Fluorescence decay spectra for FAD (350-nm excitation, 520-nm emission), thiochrome diphosphate (370-nm excitation, 435.8-nm emission), and NPR labeled enzyme (350-nm excitation, 450-nm emission). The spectra are plotted as the logarithm of the photon counts vs. time and were obtained in 0.02 M potassium phosphate (pH 7.0) at 5 °C. The best fit parameters to eq 5 and 6 are given in Table I. (A) (□) FAD (1.53 μM pyruvate dehydrogenase complex); the line is the best fit to eq 5 with τ_1 = 4.23 ns. (△) Thiochrome diphosphate (76.3 μM); the line is the best fit to eq 5 with τ_1 = 1.68 ns. (○) Pyruvate dehydrogenase complex (4.61 μM), 151 μM thiochrome diphosphate; the line is the best fit to eq 6 with τ_1 = 1.64 ns and τ_2 = 3.99. For clarity the FAD counts have been multiplied by 0.1. (B) Pyruvate dehydrogenase complex (4.61 μM), 151 μM thiochrome diphosphate; (□) oxidized FAD; (—) reduced FAD. (C) DDPM labeled enzyme complex (1.72 μM); (- - -) no ligands; (—) 58.2 μM thiochrome diphosphate; (□) 58.2 μM thiochrome diphosphate, 940 μM thiamin diphosphate. (D) NPR labeled enzyme (1.16 μM); (—) FAD oxidized; (□) FAD reduced.

acid residue on E_2. The results are summarized in the four panels of Figure 3.

The thiochrome diphosphate in 0.02 M potassium phosphate (pH 7.0) has a fluorescence decay curve (excitation 370 nm, emission 435.8 nm) which can be described in terms of a single lifetime, i.e., eq 5

$$F(t) = C_1 e^{-t/\tau_1} \tag{5}$$

where $F(t)$ is the fluorescence intensity, τ_1 is the fluorescence lifetime, and C_1 is an arbitrary amplitude parameter. However, when thiochrome diphosphate is added to the enzyme complex, two fluorescence lifetimes are needed to describe the data:

$$F(t) = C_1 e^{-t/\tau_1} + C_2 e^{-t/\tau_2} \tag{6}$$

The actual fluorescence decay curves and the best fit calculated curves using the parameters in Table I are shown in Figure 3A. The fluorescence lifetime for thiochrome diphosphate in buffer is 1.68 ns, while in the presence of the enzyme complex, about 80–85% of the fluorescence has a similar lifetime of 1.77 ns with about 15–20% having a lifetime of approximately 4 ns. The additional component in the presence of the enzyme complex may be due to excitation of the flavin which has a lifetime of 4.2 ns. The experimental fluorescence decay curve and the calculated curve for the flavin are included in Figure 3A; the fluorescence lifetimes are in Table I. Energy transfer to the flavin was examined either by reducing the flavin, which effectively removes it as a potential energy acceptor, or by displacing the thiochrome diphosphate with thiamin diphosphate (1.0 mM). The fluorescence decay curves after flavin reduction or donor displacement were compared directly with the curves obtained from the same solutions prior to reduction or displacement and to curves obtained from similar solutions containing no enzyme complex. The actual decay curves for oxidized and reduced flavin are shown in Figure 3B. A small change occurs which can be attributed to energy transfer. The decay parameters are cited in Table I. A two exponential decay is required to fit the data for both oxidized and reduced flavin. When thiochrome diphosphate is displaced by thiamin diphosphate, essentially no change in the fluorescence decay curve was observed (see Table I). This, however, is a less sensitive method of measuring energy transfer. Taking into account that a significant amount of unbound thiochrome diphosphate is present, not all binding sites are occupied (see Table I), and that the control experiments with the donor in the absence of enzyme show small changes in the fluorescence decay curves either due to hyposulfite or thiamin diphosphate presence, a small amount of energy transfer can be detected but not accurately quantitated. The fluorescence decay curve is not altered significantly by the presence of CoA (0.8 mM) and dithiothreitol (5.5 mM). The fluorescence decay curves for thiochrome diphosphate bound to the DDPM-labeled enzyme in the presence and absence of thiamin diphosphate are shown in Figure 3C, and the best fit parameters to eq 6 are summarized in Table I. No energy transfer due to the presence of DDPM or influence of DDPM on the enzyme complex with respect to transfer to flavin is present. The parameters are essentially unchanged by the presence of CoA. The lifetime decay spectrum of the DDPM modified enzyme complex in the absence of thiochrome diphosphate is also shown in Figure 3C.

The fluorescence decay curves for lipoic acid bound NPR in the presence of reduced and oxidized FAD are shown in Figure 3D, and the best fit parameters to eq 6 are summarized in Table I. Again the presence of CoA (0.8 mM)-dithiothreitol (5.5 mM) has no effect on the fluorescence decay curves. The data indicate no appreciable energy transfer is occurring.

The fluorescence decay curve for bound ANS can be adequately fit by a single exponential equation, and the fluorescence lifetime is unchanged when the lipoic acid is labeled with DDPM. Again no energy transfer occurs.

We have presented actual fluorescence decay curves in addition to the best fit parameters because in the detection of energy transfer, a direct comparison of decay curves is the most sensitive and unbiased procedure. Deconvolution of the data and fitting to four parameters (τ_1, τ_2, C_1, C_2) is a complex mathematical operation which does not give a unique description of the data. Therefore, small changes in fitting parameters cannot be interpreted unequivocally.

According to the theory for fluorescence singlet-singlet resonance energy transfer (Förster, 1946; Stryer and Haugland, 1967), the distance between an isolated donor and acceptor can be calculated from eq 7-9

$$E = R^{-6}/(R^{-6} + R_0^{-6}) \tag{7}$$

$$R_0 = 9.79 \times 10^3 (Q_D J K^2 n^{-4})^{1/6} \tag{8}$$

$$E = 1 - \tau_{D\to A}/\tau_D = 1 - Q_{D\to A}/Q_D \tag{9}$$

where E is the efficiency of energy transfer, R is the distance between donor and acceptor, R_0 is the distance for which E =

TABLE I: Fluorescence Lifetime Parameters for Enzyme-Associated Molecules.

Donor	Acceptor	C_1	τ_1	C_2	τ_2	$(L_B)/(L_F)$[a]	θ_D[b]
Thiochrome diphosphate	FAD (oxidized)	0.887	1.64	0.113	3.99	1.98	0.77
	FAD (reduced)	0.842	1.78	0.158	4.10		
Thiochrome diphosphate	FAD (oxidized)	0.784	1.77	0.216	3.62	0.24[c]	0.001
	FAD (oxidized)	0.925	1.69	0.075	4.22	1.87[d]	0.76
Thiochrome diphosphate	DDPM-lipoic acid	0.949	1.72	0.051	4.66	1.08	0.65
	Lipoic acid	0.958	1.71	0.042	4.88	1.08	0.65
	DDPM-lipoic acid	0.984	1.58	0.016	4.48	0.11[c]	0
	DDPM-lipoic acid	0.925	1.69	0.075	4.22	1.23[d]	0.75
Thiochrome diphosphate[e]	None	1	1.68				
ANS	DDPM-lipoic acid	1	16.69			0.08	0.98
	Lipoic acid	1	16.65			0.08	0.98
NPR-lipoic acid	FAD (oxidized)	0.571	24.1	0.429	68.7		1
	FAD (reduced)	0.634	23.4	0.366	72.0		1
	FAD (oxidized)	0.900	43.5	0.100	132.2	*d*	1
FAD[f]	None	1	4.23				
FAD[g]	None	1	4.41				

[a] Ratio of the amount of donor bound to the enzyme to the amount free in solution. [b] θ_D is the fraction of donor sites occupied per 4.6×10^6 molecular weight protein (the maximum is 24 for thiochrome diphosphate, 48 for PM). [c] 1.0 mM thiamin diphosphate added to displace thiochrome diphosphate. [d] 0.8 mM CoA and 5.5 mM dithiothreitol added. [e] Determined in the absence of enzyme. [f] 350-nm excitation, 520-nm emission. [g] 450-nm excitation, 520-nm emission.

0.5, Q_D and τ_D are the quantum yield and fluorescence lifetime of the donor in the absence of the acceptor, $Q_{D\rightarrow A}$ and $\tau_{D\rightarrow A}$ are the quantum yield and fluorescence lifetime of the donor in the presence of the acceptor, J is the overlap integral of the corrected donor emission spectrum and the acceptor absorbance spectrum, n is the refractive index of the medium, and K^2 is the dipole orientation factor. Values of R_0 were calculated for the donor-acceptor pairs under investigation using the measured values for Q_D, the calculated values for J (Cantley and Hammes, 1975), and assuming $K^2 = 2/3$ and $n = 1.4$. The values obtained are as follows: thiochrome diphosphate-DDPM, 28 Å; thiochrome diphosphate-FAD, 31 Å (Moe et al., 1974); ANS-DDPM, 33 Å; and NPR-FAD, 27 Å. A small amount of energy transfer is observed only with the donor-acceptor pair thiochrome diphosphate-FAD. Assuming the energy transfer efficiency is about 5%, the calculated distance between thiochrome and FAD is 51 Å, in good agreement with a previous determination (47 Å) utilizing a phase difference method for determining the fluorescence lifetimes (Moe et al., 1974). For the other donor-acceptor pairs, no energy transfer is observed. Assuming that an energy-transfer efficiency of 10% or greater would be detected (which is a generous estimate) permits calculation of lower bounds for the distances between the donor and acceptor. The values obtained are as follows: thiochrome diphosphate-DDPM, 40 Å; NPR-FAD, 39 Å; and ANS-DDPM, 47 Å.

Discussion

The most precise measurement of the incorporation of maleimides into the enzyme complex is obtained with radioactive NEM. The labeling may be somewhat incomplete since about 6% of the enzyme activity remains after labeling. On the other hand, about 5% of the label is in the E_3 component. Therefore the maximum number of NEM that can be incorporated into E_2 is probably close to the measured value, 47 per 4.6×10^6 molecular weight with an estimated uncertainty of ±5%. About the same number is obtained with DDPM (40) and NPR (46). The former is somewhat uncertain because the extinction coefficient is not known precisely for the DDPM modified enzyme complex and is small relative to the enzyme complex extinction coefficient at 450 nm. For NPR, the extinction coefficient is also somewhat uncertain. The fluorescence emission spectrum of NPR labeled enzyme complex has an increased intensity in the region of 450 nm which is most likely due to excimer fluorescence of pyrene. Either a small fraction of doubly labeled lipoic acid groups or strongly interacting singly labeled lipoic acid moieties within the complex could account for this fluorescence. The evidence suggesting the lipoic acid is specifically labeled by NEM in the presence of pyruvate and thiamine diphosphate has been presented by others (Grande et al., 1975; Danson and Perham, 1976). Presumably an acetyl group is transferred to lipoic acid by reduction of the disulfide by the hydroxyethyl adduct. The maleimide can then react with the free sulfhydryl group of lipoic acid. The possibility exists that the maleimide also can displace the *S*-acetyl group, although this does not seem likely. Thus in principle either one or two maleimides may be incorporated per lipoic acid, with the former being more probable. In terms of the subunit stoichiometry proposed by Reed et al. (Eley et al., 1972), the results obtained suggest either 48 (or less likely 24) lipoic acid groups are present per 4.6×10^6 molecular weight protein.

The number of lipoic acid residues present per 4.6×10^6 molecular weight has been reported to be 38.6 (Koike et al., 1960) and more recently 24-26 (Eley et al., 1972). While the work reported here was in progress, Danson and Perham (1976) reported two lipoic acids per 80 000 molecular weight of the E_2 polypeptide chain using radioactive NEM labeling and assuming one NEM is incorporated per lipoic acid. However, the number of E_2 polypeptide chains present per enzyme complex was not specified. Using the data presented, we calculate 32 lipoic acids per 4.6×10^6 molecular weight protein. The subunit stoichiometry of the *E. coli* pyruvate dehydrogenase complex is still controversial (Bates et al., 1975; Perham and Cooper, 1977; Vogel, 1977). The binding results reported here and those for other specific ligands we have observed (Moe and Hammes, 1974; Shepherd and Hammes, 1976; Shepherd et al., 1976) are most consistent with the

TABLE II: Distances between Fluorescence Donors and Acceptors on the Pyruvate Dehydrogenase Multienzyme Complex.

Donor (functional site)	Acceptor (functional site)	R (Å)
Thiochrome diphosphate (E_1, catalytic)	FAD (E_3, catalytic)	47,[a] ~51
	DDPM (E_2, lipoic acid)	≥40
ANS (E_1, regulatory)	FAD (E_3, catalytic)	>58[b]
	DDPM (E_2, lipoic acid)	≥47
NBM[f] (E_1, sulfhydryl)	ANS (E_1, regulatory)	≥35[c]
	Thiochrome diphosphate (E_1, catalytic)	≥42[c]
Thiochrome diphosphate (E_1, catalytic)	DDPM (E_1, sulfhydryl)	≥39[c]
ANS (E_1, regulatory)	DDPM (E_1, sulfhydryl)	49[c]
NPR (E_2, lipoic acid)	FAD (E_3, catalytic)	≥39
ANM[g] (E_2, lipoic acid)	FAD (E_3, catalytic)	≥40[d]
εCoA (E_2, catalytic)	FAD (E_3, catalytic)	≥50[e]

[a] Moe et al., 1974. [b] Shepherd and Hammes, 1976. [c] Papadakis and Hammes, 1977. [d] Grande et al. (1976) estimated assuming R_0 ~30 Å and an energy transfer efficiency of ≤0.1. [e] Shepherd et al., 1976. [f] *N*-[*p*-(2-benzoxazolyl)phenyl]maleimide. [g] *N*-(1-anilinonaphthalyl-4)maleimide.

subunit stoichiometry proposed by Eley et al. (1972): 24:24:12 for E_1:E_2:E_3.

Also while this work was in progress, Grande and co-workers (1976) reported the labeling of lipoic acid with the fluorescent compound *N*-(1-anilinonaphthalyl-4)maleimide, which has fluorescent properties similar to DPNH. In addition to labeling under conditions similar to those used in this work, the labeling was done in the presence of DPNH rather than pyruvate and thiamin diphosphate. We have also carried out labeling with NPR under similar conditions except Sephadex G-25, ammonium sulfate precipitations, and dialysis were used to eliminate the DPNH and excess NPR from the labeled enzyme rather than Sephadex G-10. The overall activity of the labeled complex was about 20%, and the complex showed a larger fluorescence at 450 nm (345-nm excitation) than found using the alternative labeling procedure. It is not clear whether this increased fluorescence is due to more extensive labeling (two labels per lipoic acid could yield appreciable 450-nm excimer fluorescence) in spite of the significant remaining activity or is due to incorporation of some DPNH.

The binding of εCoA to the CoA displaceable sites on the DDPM labeled enzyme complex is characterized by a dissociation constant approximately four times greater than found with the native enzyme. This indicates that the lipoic acid and CoA binding sites interact. Unfortunately the large dissociation constant of εCoA made it impossible to carry out a meaningful energy-transfer experiment with enzyme bound εCoA as the energy donor and DDPM-labeled lipoic acid as the energy acceptor.

On the one hand, the binding of thiochrome diphosphate or ANS to E_1 is not altered significantly by the DDPM label being on E_2 or by the binding of CoA to E_2. The fluorescent properties of thiochrome diphosphate and ANS also are unchanged. Conversely the binding of pyruvate and thiamin diphosphate to E_1 and the reduction of FAD on E_3 has no effect on the fluorescent properties of PM-labeled lipoic acid. Taken together, these results suggest the absence of appreciable interactions between the catalytic sites of the three enzymes and between the acetyl-CoA regulatory site of E_1 and the catalytic sites of E_2 and E_3.

The major uncertainty in interpreting the energy-transfer measurements is the assessment of the steric factor K^2. However, both the thiochrome diphosphate and NPR fluorescence have a polarization significantly different from their limiting polarizations. Therefore, assuming K^2 to have the value associated with a freely rotating donor and acceptor cannot be appreciably in error. Upper and lower limits for K^2 have been estimated using the measured polarizations of the energy donor and assuming the energy acceptor does not rotate (Dale and Eisinger, 1974; Zukin et al., 1977). These extreme estimates indicate the *maximum* uncertainty in the calculated distances is about 40%; the probable uncertainty is considerably less than this. Furthermore, the large number of donor-acceptor pairs which have been used to study the *E. coli* pyruvate dehydrogenase complex all give essentially the same result, little or no energy transfer. It is unlikely K^2 is close to zero for all these cases. The donor-acceptor pairs, the functional locations of the energy donors and acceptors, and the minimum distances between the donors and acceptors which have been determined for the enzyme complex are summarized in Table II. The essential point to note is that the catalytic sites of the three enzymes are at least 40 Å apart. This is not consistent with a mechanism in which a single lipoic acid rotates between the three catalytic sites. The lack of energy transfer to and from lipoic acid derivatives also rules out a mechanism involving the transfer of intermediates between two lipoic acids. For this mechanism half of the lipoic acid residues should be within 28 Å of the catalytic site of E_1 and half within 28 Å of the catalytic site of E_3 which should result in an energy-transfer efficiency of about 0.25 for the probes used. This assumes all of the lipoic acid residues are labeled which appears to be approximately true. The possibility exists that the conformation of the enzyme complex during catalysis differs from that during energy-transfer measurements, but this does not seem likely.

Several alternative mechanistic possibilities exist. The energy-transfer measurements indicate only an *average* distance between acceptors and donors. If only a small fraction of the catalytic sites actually takes part in the overall reaction, then the observed energy-transfer efficiency could be anomalously low. For the single rotating lipoic acid mechanism a measured energy-transfer efficiency of 0.10 or less for energy transfer between lipoic acid and the catalytic sites of E_1 and E_3 would require 20% or less of the catalytic sites (or lipoic acids) to participate in catalysis of the overall reaction; for the mechanism involving two lipoic acids 40% or less of the lipoic acids would have to participate in the overall reaction. This assumes the mechanism requires the maximum distance between lipoic acid and the catalytic sites to be 28 Å with $R_0 \approx 28$ Å. This second possibility, namely, that about half of the lipoic acids are not coupled to all three enzymes and that two lipoic acids participate in transfer of the intermediates between E_1 and E_3, is also consistent with the lack of significant energy transfer between enzyme bound εCoA and FAD (since only half of these sites would be functionally coupled and fully extended εCoA is about 30 Å in length) and with the NPR excimer formation suggesting some of the lipoic acid residues are close to each other. The participation of more than two lipoic acids in a single catalytic cycle also would be consistent with the energy-transfer measurements. Still another possibility is that the coupling of the catalytic sites on E_1 and E_3 to the lipoic acid occurs via other groups on the protein and/or conformational changes, thus permitting the lipoic acid to be further than 28 Å from the catalytic sites of E_1 and E_3. Future experiments will be designed to delineate these and other mechanistic possibilities.

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Pyridoxamine-Pyruvate Transaminase. 1. Determination of the Active Site Stoichiometry and the pH Dependence of the Dissociation Constant for 5′-Deoxypyridoxal†

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ABSTRACT: Spectrophotometric titration of pyridoxamine-pyruvate transaminase (EC 2.6.1.30) with pyridoxal at pH 7.15 gives four equivalent binding sites per tetramer. The pH dependence of the equilibrium constant for the association of 5′-deoxypyridoxal with the active site lysine residue was determined spectrophotometrically. These dissociation constants increase with increasing pH over the range pH 7.5–9 and are correlated with the values obtained from fast reactions kinetics (Gilmer, P. J., and Kirsch, J. F. (1977), *Biochemistry 16* (following paper in this issue)). In addition to this specific reaction at an active site lysine residue, a second slower reaction at non-active site residues is observable at pH values greater than 8. The pH dependencies of the association and dissociation rate constants for this slow reaction were studied over the pH range 8 to 9 after blocking the active site by $NaBH_4$ reduction of the pyridoxal adduct. The enzyme is stabilized and markedly activated by potassium ion.

Pyridoxamine-pyruvate transaminase (EC 2.6.1.30) (PPT)[1] is unique among pyridoxyl dependent transaminases in that the vitamin B-6 derivative acts not as a cofactor but is itself a substrate in the enzymatic reaction shown in Scheme I. Wada and Snell (1962) isolated PPT from *Pseudomonas* sp. MA-1 grown on pyridoxamine as the source of carbon and nitrogen. This enzyme is part of the degradative pathway for B-6 compounds utilized by this organism. The enzyme was charac-

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[1] Abbreviations used are: 5′-deoxy-PL, 5′-deoxypyridoxal; PPT, pyridoxamine-pyruvate transaminase; PL, pyridoxal; PM, pyridoxamine; PLP, pyridoxal 5′-phosphate.