Fluorescence Energy-Transfer Measurements between Coenzyme A and Flavin Adenine Dinucleotide Binding Sites of the *Escherichia coli* Pyruvate Dehydrogenase Multienzyme Complex†

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ABSTRACT: The interaction of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* with 1,N^6-etheno-CoA (ϵCoA) and coenzyme A (CoA) has been investigated using equilibrium binding, steady-state fluorescence, and fluorescence lifetime measurements. A procedure for the resolution of the pyruvate dehydrogenase multienzyme complex into the pyruvate dehydrogenase enzyme and the transacetylase–flavoprotein subcomplex also is given. Direct binding studies with ϵCoA indicate that 25 bound ϵCoA molecules/multienzyme complex can be readily displaced by CoA, while approximately 21 bound ϵCoA molecules/transacetylase–flavoprotein subcomplex can be displaced by CoA. The dissociation constant for the CoA displaceable ϵCoA is 57.8 μM for the complex and 126 μM for the subcomplex in 0.02 M potassium phosphate (pH 7.0) at 5 °C. The kinetic behavior of ϵCoA as a substrate was investigated and compared with that of CoA under a variety of conditions; the apparent Michaelis constants for ϵCoA are considerably larger than those for CoA, while the corresponding maximal velocities are smaller. Fluorescence energy transfer measurements between bound ϵCoA on the dihydrolipoyl transacetylase enzyme and flavin adenine dinucleotide on the dihydrolipoyl dehydrogenase enzyme either in the complex or subcomplex indicate, assuming the emission and absorption dipoles are randomly oriented, that these two probes must be at least 50 Å apart.

The purified pyruvate dehydrogenase multienzyme complex from *Escherichia coli* has been shown to contain three component enzymes which catalyze the decarboxylation of pyruvate and the formation of acetyl-CoA (Koike and Reed, 1960a). The pyruvate dehydrogenase (E_1)[1] contains regulatory binding sites for acetyl-CoA and GTP and the catalytic binding sites for pyruvate and thiamin diphosphate. The lipoyl transacetylase (E_2) contains the binding sites for CoA and lipoic acid linked to 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 studies and chemical evidence, in which the 24 polypeptide chains of E_1, the 24 polypeptide chains of E_2, and the 12 polypeptide chains of E_3 are arranged such that the catalytic sites of the three components are within a sphere of 28-Å diameter with the radius defined by the lipoic acid–lysine prosthetic group (Koike and Reed, 1960b). The lipoic acid group is postulated to transfer intermediates between active sites during the course of catalysis. A modification of this mechanism proposes an exchange of intermediates between lipoic acids; this doubles the lipoic acid requirement and expands the sphere containing the three catalytic sites to 56 Å (Koike et al., 1963).

Previous works using thiochrome diphosphate, a TPP analogue, and ANS bound to E_1 as fluorescence energy-transfer donors to FAD on E_3 have suggested the ligand binding sites on E_1 are greater than 47 Å for TPP and greater than 58 Å for ANS from the FAD (Moe et al., 1974; Shepherd and Hammes, 1976). The ANS has been found to be a competitive inhibitor of acetyl-CoA binding to E_1. The major uncertainty in these distances arises from the inability to ascertain the relative orientation of the donor–acceptor pairs. Although this is probably not a major source of error, in an effort to circumvent this problem, ligand–ligand site distances are being determined by singlet–singlet fluorescence energy-transfer measurements utilizing a variety of donor–acceptor pairs; it is unlikely that unfavorable orientations will distort the calculated distances for all pairs studied. From the results obtained thus far, it seems likely that the flavin on E_3 is quite distant ($\geq$50 Å) from the catalytic and acetyl-CoA binding sites on E_1.

The work presented here is a continuation of these specific site to site energy-transfer measurements. The efficiency of singlet–singlet fluorescence energy transfer from a fluorescent donor molecule to an absorbing acceptor molecule is defined by eq 1

$$E = 1 - Q_{D\rightarrow A}/Q_D = 1 - \tau_{D\rightarrow A}/\tau_D \quad (1)$$

where Q and τ symbolize the quantum yield and fluorescence lifetime of the donor molecule, and the subscripts D→A and D refer to measurements made in the presence of the acceptor and its absence, respectively. The transfer efficiency is related to the distance, R, between an isolated donor–acceptor pair by eq 2

$$E = R^{-6}/(R^{-6} + R_0^{-6}) \quad (2)$$

where R_0 is the distance of separation for which $E = 0.5$ and can be calculated using eq 3 (Förster, 1965; Stryer and Haugland, 1967).

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

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; TPP, thiamin diphosphate; E_1, pyruvate dehydrogenase; E_2, dihydrolipoyl transacetylase; E_3, dihydrolipoyl dehydrogenase; E_2–E_3, pyruvate dehydrogenase subcomplex with E_1 removed; ϵCoA, 1,N^6-etheno-coenzyme A; tricine, *N*-tris(hydroxymethyl)methylglycine; GTP, guanosine triphosphate; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD.

In this equation n is the refractive index, K^2 is the mutual emission-absorption dipole orientation factor for the two moieties, and J is the integral of the spectral overlap for the normalized donor fluorescence and acceptor absorption spectra.

In this study, 1,N^6-etheno-CoA (εCoA) was used as the donor moiety and FAD as the acceptor. The results obtained suggest that the εCoA, which can be bound specifically on E_2, and the FAD on E_3 are at least 50 Å apart. The equilibrium binding and steady-state kinetic parameters characterizing the interaction of CoA and εCoA with the complex and the E_2-E_3 subcomplex also were determined.

Materials and Methods

Chemicals. The εCoA was obtained from P.L. Biochemicals as the lithium salt, the quinine bisulfate from Aldrich, and all other biochemicals were purchased from Sigma. The purity of the εCoA was checked by analytical thin-layer and paper chromatography using ultraviolet light for detection. With ethanol-1.0 M ammonium acetate, pH 7.5 (7:3), or isobutyric acid-NH_3-H_2O (75:1:24), CoA and εCoA have identical R_f values. Any CoA impurity in the εCoA is masked by the fluorescence of εCoA. The only observed impurities that absorbed ultraviolet light or fluoresced were oxidized εCoA and CoA. The addition of 50 mM (fivefold excess) cysteine or 50 mM dithiothreitol to the stock ligand solutions essentially eliminated this oxidation. The concentration of CoA was determined spectrophotometrically using an extinction coefficient of 16840 M^{-1} cm^{-1} at 258 nm (Stadtman, 1957), and the concentration of εCoA was determined using the extinction coefficients for 1,N^6-etheno-ATP of 4900, 5700, 5600, and 2900 M^{-1} cm^{-1} at the wavelengths 258, 265, 275, and 294 nm (Secrist et al., 1972). All measurements were corrected for buffer and dithiothreitol absorption. An estimate of the amount of CoA impurity in εCoA was made by assuming that any deviation in the ratios of the absorbances at 294 to 258, 275 to 258, and 265 to 258 nm from that of the known 1,N^6-etheno-ATP spectrum (Secrist et al., 1972) was due to CoA contamination. By this method an upper bound on the CoA concentration was found to be 4-10%. The CoA and εCoA solutions were prepared fresh daily and stored at 5 °C with 13 mM dithiothreitol in 0.02 M potassium phosphate (pH 7.0) to minimize oxidation. All other chemicals used were the best available commercial grades, and deionized distilled water was used in all solutions.

Pyruvate Dehydrogenase Complex and Subcomplexes. The preparation, purification, activity, and protein analyses of the enzyme complex were as previously described (Reed and Willms, 1966; Schwartz et al., 1968). The complexes with 70% of the FAD removed and >99% of the TPP removed also were prepared as previously described (Shepherd and Hammes, 1976). The enzyme complex with over 99% of the TPP removed again will be referred to as the apo complex.

The E_1 and E_2-E_3 enzymes were resolved by chromatography on a Sepharose 6B (Pharmacia) column at pH 9.0 (0.02 M ethanolamine-0.02 M tricine). The apo complex (about 150 mg in 4.5 ml of 0.02 M potassium phosphate, pH 7.0) was dialyzed for 1 h at 4 °C against 500 ml of 0.02 M ethanolamine-0.02 M potassium phosphate (pH 9.5). The protein then was applied to the Sepharose 6B column (2.5 cm i.d. × 90 cm), the flow rate was adjusted to 20 ml/h, and 4.0-ml fractions were collected. A typical elution pattern is shown in Figure 1. The first peak contained the E_2-E_3 subcomplex; the second contained E_1 and a small amount of subcomplex. The pooled fractions for each peak were dialyzed for 10 h at 4 °C against

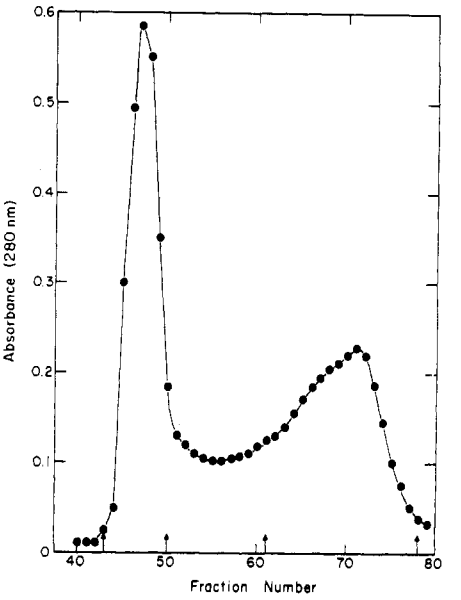

FIGURE 1: Elution profile of E_2-E_3 and E_1 for the apopyruvate dehydrogenase complex resolution on Sepharose 6B at pH 9.0 (0.02 M ethanolamine-0.02 M tricine), 5 °C. The effluent was monitored at 280 nm. The fraction size was 4 ml and the flow rate 20 ml/h. Fractions 43-50 were pooled as E_2-E_3, and fractions 61-78 were pooled as E_1.

2 l. of 0.05 M potassium phosphate (pH 7.0). The E_1 fractions were further purified by ammonium sulfate fractionation in 0.05 M potassium phosphate (pH 7.0): 50% saturated ammonium sulfate precipitated essentially all of the E_2-E_3 contamination and some E_1; a second precipitation with 70% saturated ammonium sulfate yielded essentially pure E_1. The E_2-E_3 subcomplex was preferentially precipitated by centrifugation at 4 °C for 4.5 h at 75 000g. Finally the E_1 and E_2-E_3 precipitates were suspended in 0.05 M potassium phosphate (pH 7.0) and dialyzed for 24 h at 4 °C against the same buffer. The stock solutions were clarified by centrifugation at 19 000g for 15 min. The specific activity of the pyruvate dehydrogenase component (E_1) as measured by the ferricyanide assay was essentially the same as found for the intact complex, 20-25 μmol of pyruvate/mg-h. The purity of the E_1 and E_2-E_3 fractions was estimated from enzymatic assays of each fraction using both ferricyanide and NADH assays, and from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The E_2-E_3 subcomplex contamination of E_1 is <1%, while the E_1 contamination of the E_2-E_3 subcomplex ranged from 4 to 9%.

Binding Measurements. The binding of εCoA to the apo complex and E_2-E_3 subcomplex was studied by the technique of forced dialysis at 4 °C (Cantley and Hammes, 1973). Measurements were completed within 60 min to minimize ligand decomposition. PM 30 ultrafiltration membranes (Amicon) were used to exclude all protein. Because these membranes must be used damp, approximately a 3-μl dilution of the ligand occurs in the first sample forced through the membrane. Therefore, three 25-30-μl aliquots were collected, and the last two were used to determine the free ligand concentration. The fluorescence of the samples (305-nm excitation, 405-nm emission) was used to measure the ligand concentrations. At low concentrations of εCoA, the protein fluorescence and light scattering at the εCoA emission wavelength do not permit an accurate determination of the concentration. Therefore, the total ligand concentration was determined by preparing a sample containing all components except the protein and measuring the fluorescence of aliquots of this so-

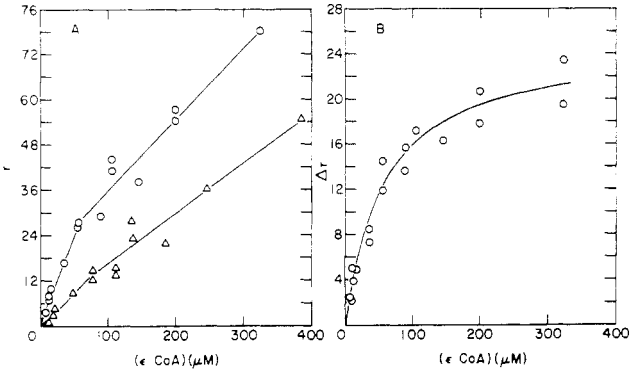

FIGURE 2: (A) A plot of r vs. (ϵCoA), where r is the number of moles of ϵCoA bound per mole of apopyruvate dehydrogenase complex and (ϵCoA) is the concentration of free ϵCoA. The data were obtained by forced dialysis at 5 °C: (○) 1.65–2.47 μM apo complex, 10–430 μM ϵCoA; (△) 1.65–2.47 μM apo complex, 10–430 μM ϵCoA, and 600–800 μM CoA. All solutions contained 2.2 mM Mg^{2+}, 1.17 mM dithiothreitol, and 0.02 M potassium phosphate (pH 7.0). The data plotted are the averaged duplicates; the lines have no theoretical significance. (B) A plot of Δr vs. (ϵCoA), where Δr is the number of moles of ϵCoA bound per mole of apo complex that are displaceable by saturating CoA (600–800 μM), and (ϵCoA) is the concentration of free ϵCoA found in the absence of CoA. The data are from Figure 2A. The line is a least-squares fit using eq 4 with n = 25.2 and K_D = 57.8 μM.

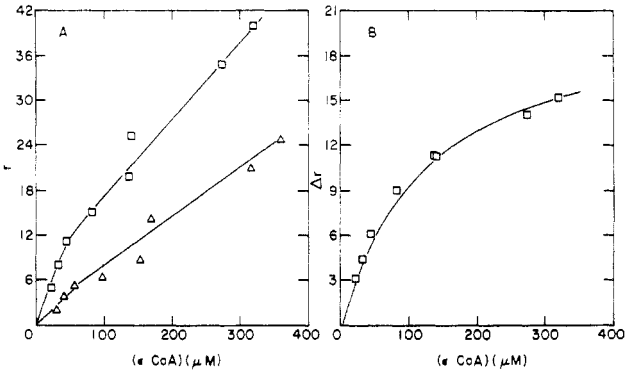

FIGURE 3: (A) A plot of r vs. (ϵCoA), where r is the number of moles of ϵCoA bound per mole of E_2–E_3 subcomplex, and (ϵCoA) is the concentration of free ϵCoA. The data were obtained by forced dialysis at 5 °C: (○) 1.45–2.90 μM E_2–E_3 subcomplex and 10–435 μM ϵCoA; (△) 1.45–2.90 μM E_2–E_3, 10–435 μM ϵCoA, and 600–800 μM CoA. All solutions contained 2.2 mM Mg^{2+}, 1.17 mM dithiothreitol, and 0.02 M potassium phosphate (pH 7.0). The data plotted are the averaged duplicates; the lines have no theoretical significance. (B) A plot of Δr vs. (ϵCoA), where Δr is the number of moles of ϵCoA bound per mole of subcomplex that are displaceable by saturating CoA (600–800 μM), and (ϵCoA) is the concentration of free ϵCoA found in the absence of CoA. The data are from Figure 3A. The line is a least-squares fit using eq 4 with n = 21.2 and K_D = 126 μM.

lution in 0.02 M potassium phosphate (pH 7.0) at 25 °C. Dithiothreitol (300 μM) was present in all solutions because ϵCoA can be oxidized to dimers in the absence of a reducing agent with a resultant slow quenching of the ϵCoA fluorescence. Inner filter effects of ϵCoA absorbance were negligible, as the concentration of the ligand was always less than 10 μM in the solution on which fluorescent measurements were made. With the optical settings held constant through a given set of measurements, the ligand concentration was assumed to be linear with fluorescence. In order to measure the amount of ϵCoA specifically bound at the CoA binding site, 600–800 μM CoA was added to displace bound ϵCoA, and the amount of "nonspecific" ϵCoA binding was determined by forced dialysis experiments, as described above.

Spectroscopic Measurements. Ultraviolet and visible absorption measurements were made with a Zeiss PMQ II or with a Cary 14 spectrophotometer. Steady-state fluorescence 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 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 305 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 use of 305 nm as the excitation wavelength, the inner filter effects associated with absorption of exciting light and of emitted fluorescence, and the anisotropy of fluorescent species (Shinitzky, 1972).

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 5 °C, and dry N_2 was circulated through the sample chamber. An interference filter (Ditric) with maximum transmittance at 410 nm (9.7-nm band-pass) was used in the emission path. The excitation wavelength was fixed either by a monochromator at 305 nm (10-nm band-pass) or by an interference filter with maximum transmittance at 313 nm (12-nm band-pass). All nanosecond decay spectra were accumulated for the same analysis time. Scattering spectra of the enzyme (100% or 30% FAD content) in the absence of ϵCoA were accumulated to be used as blanks. The treatment of the data and fitting procedures were as described before (Shepherd and Hammes, 1976).

Results

The results of the measurements of ϵCoA binding to the apopyruvate dehydrogenase complex and to the apo complex in the presence of saturating CoA in 0.02 M potassium phosphate (pH 7.0) at 5 °C are summarized in Figure 2A as a plot of r, the moles of ligand bound per mol of enzyme complex, vs. the free ϵCoA concentration. In calculating r, a molecular weight of 4.6 million is assumed for the enzyme complex and a molecular weight of 2.4 million for the E_2–E_3 subcomplex (Eley et al., 1972). The samples used to determine these data contained 1.65–2.47 μM apo complex, 2.2 mM Mg^{2+}, 1.17 mM dithiothreitol, 10–430 μM ϵCoA, and in experiments with saturating CoA, 600–800 μM CoA.

The ϵCoA obviously binds to a larger number of sites on the enzyme than occupied by CoA. However, a binding isotherm for specific binding at the CoA site can be constructed from the data by plotting Δr vs. the free ligand concentration (Figure 2B) where Δr is defined as the difference in r values for ϵCoA binding with and without a saturating concentration of CoA at the same total ϵCoA and apo complex concentration.

The data in Figure 2B were fit by a nonlinear weighted least-squares analysis to eq 4

$$\Delta r = \frac{n(L)}{K_D + (L)} \tag{4}$$

which assumes n independent binding sites, characterized by the dissociation constant K_D, and L is the free ligand concentration. The results obtained gave n = 25.2 sites and K_D = 57.8 μM; the curve in Figure 2B was calculated with these parameters and eq 4.

The results of the binding of ϵCoA to the E_2–E_3 subcomplex

TABLE I: Steady-State Parameters for CoA and εCoA.[a]

		CoA			εCoA		
pH	T (°C)	V_{max}[d]	K_m (μM)	C_l[e] (μM)	V_{max}[d]	K_m (μM)	C_l[e] (μM)
8.0[b]	30	29.1	2.8	80	10.8	257	>350
7.0[c]	30	14.7	2.5	20	13.9	57	115
8.0[b]	5	8.60	1.4	25	4.77	61	>350
7.0[c]	5	3.58	1.0	25	2.77	8	46

[a] 0.14 nM enzyme complex, 2.9 mM NAD, 430 μM TPP, 1.5 mM dithiothreitol, 5.14 mM pyruvate, 400 μM Mg^{2+}. [b] 0.1 M potassium phosphate. [c] 0.02 M potassium phosphate. [d] μmol of NADH formed/min-mg complex. [e] Concentration above which $1/v$ vs. $1/(S)$ plots become nonlinear.

in 0.02 M potassium phosphate (pH 7.0) at 5 °C are summarized in Figure 3 as a plot of r vs. the free ligand concentration in the presence and absence of saturating CoA (3A) and as a plot of Δr vs. the free ligand concentration (3B). The samples contained 1.45–2.90 μM E_2–E_3, 2.2 mM Mg^{2+}, 1.17 mM dithiothreitol, 10–435 μM εCoA, and in experiments with saturating CoA, 600–800 μM CoA. A least-squares fit of the data in Figure 3B according to eq 4 gave n = 21.2 sites and K_D = 126 μM; the curve shown in Figure 3B was calculated using these parameters and eq 4.

The data used to derive the plots in Figures 2 and 3 are subject to errors in the determination of each r of ±5–10% and for each free ligand concentration of ±5%. The resultant error in a Δr value is then ±10–20%. Repeated determinations were averaged only if the difference in Δr values was less than 20%.

Steady-State Kinetics with εCoA and CoA as Substrates. The apparent Michaelis constants and maximal velocities with CoA and εCoA as substrates were determined using the NADH assay with the following components held constant: 0.11 or 0.14 nM apo complex, 2.9 mM NAD, 430 μM TPP, 1.5 mM dithiothreitol, 5.14 mM pyruvate, and 400 μM Mg^{2+}. Measurements were carried out at 30 and 5 °C in 0.1 M potassium phosphate (pH 8.0) and 0.02 M potassium phosphate (pH 7.0). The reaction was initiated by the addition of CoA or εCoA and monitored by following the change in absorbance at 340 nm. The amount of CoA added was varied from 1 to 150 μM; the εCoA added was varied from 5 to 350 μM. With both CoA and εCoA, the initial velocity of the reaction was strongly inhibited at high coenzyme concentrations. The apparent Michaelis constants and maximal velocities were determined from a weighted least-squares analysis of the linear region of plots of $1/v$ vs. $1/(S)$, where v is the initial steady state velocity and (S) is the substrate concentration. The results obtained are summarized in Table I. The values tabulated, because of the strong substrate inhibition, can be regarded as lower bounds of the maximal velocity and upper bounds of the K_M.

Using the NADH assay, the percent conversion of CoA or εCoA to acetyl-CoA or ε-acetyl-CoA was determined by allowing the NADH assay to go to completion. The samples were prepared as in the Michaelis constant determinations with 7.1 nM enzyme complex in 0.1 M potassium phosphate (pH 8.0) at 30 °C. The total change in optical density at 340 nm was converted to an apparent NADH concentration (ϵ_{340} = 6220 M^{-1} cm^{-1}) (Kornberg and Horecker, 1953). The percent conversion was defined as the apparent concentration of NADH formed divided by the total NADH concentration possible if all the CoA or εCoA were converted to product. For CoA, the conversion was 97–99%; for εCoA, the conversion was 77–80%. Since there is at most a 10% CoA contamination in the εCoA, the CoA conversion could account for only 10% of the observed absorbance change in the assay. Thus εCoA must be a substrate of the enzyme complex and not a modifier of the CoA kinetics. The fact that only 80% of the εCoA apparently acts as a substrate suggests some nonreactive impurities may be present.

Fluorescence Measurements. The corrected fluorescence emission spectrum of εCoA with an excitation wavelength of 305 nm has a maximum at 410 nm which is unaltered in position or spectral shape on binding to the apo complex or E_2–E_3 subcomplex. An absorbance difference spectrum of εCoA bound to the apo complex (Δr = 20.7, 0.33 μM apo complex, 590 μM dithiothreitol, 330.7 μM εCoA, in 0.02 M potassium phosphate at pH 7.0, 5 °C) showed no change in extinction coefficient, $\Delta\epsilon$, at wavelengths greater than 295 nm and had a single minimum of $\Delta\epsilon = -5100$ M^{-1} cm^{-1} at 275 nm.

The quantum yield of 7 μM εCoA in 0.02 M potassium phosphate (pH 7.0), 2.2 mM Mg^{2+}, and 1.17 mM dithiothreitol at 5 °C was determined to be 0.485 ± 0.018 (305-nm excitation) by comparison with 6.8 μM quinine bisulfate in 0.1 N H_2SO_4 at 25 °C. This value when corrected for anisotropy effects (+3%) (Shinitzky, 1972) and the shift in excitation wavelength from 345 to 305 nm (+2%) (Chen, 1967) is 0.509. This value is unchanged (within ±5%) when εCoA binds to the apo complex or E_2–E_3 with Δr in the range 12–18 and the ratio of bound to free εCoA of 0.16 to 0.21. The largest change in εCoA fluorescence observed on displacement of εCoA by CoA (Δr changes from 17.9 to 0, r_{final} = 23) was +4%. Nanosecond lifetime measurements on similar samples at 5 °C showed no change in fluorescence lifetime, τ, from the value for εCoA in the absence of protein (τ = 25.0 ns ± 0.5 ns). The same lifetime was observed for both εCoA bound to E_2–E_3 with 100% FAD with and without saturating CoA and to the apo complex (100% or 30% FAD content) without CoA. The lifetime decay spectra were superposable, and all were best fit by a single exponential decay. The intrinsic error in the lifetime measurements and the background fluorescence of unbound εCoA and εCoA bound to non-CoA displaceable sites gives an uncertainty in the fluorescence lifetime of the εCoA bound to CoA sites of about ±10%.

The measurement of steady-state fluorescence polarization of εCoA bound to the apo complex is complicated by the εCoA nonspecifically bound to the complex. The polarization of free εCoA is negligibly small; the polarization of the nonspecifically bound εCoA also was found to be negligible by measuring the polarization of εCoA bound in the presence of saturating CoA. In the case of both unbound and nonspecifically bound εCoA having negligible polarizations, the polarization of the specifically bound probe with all specific sites saturated, P_B, can be found using eq 5

$$1/P = 1/P_B + \left[\frac{(I_V + I_H)_F}{(I_V + I_H)_B}\right][(L_F)/(L_B)](1/P_B) \quad (5)$$

where $(I_V + I_H)_F$ and $(I_V + I_H)_B$ represent the sum of vertically and horizontally polarized intensities emitted by the free ligand and bound ligand, respectively, L_F is the total free and nonspecifically bound ligand concentration, and L_B is the specifically bound ligand concentration (Cantley and Hammes, 1975). The polarization of the specifically bound εCoA was found by titrating the enzyme apo complex with εCoA at 5 °C (7.14 μM apo complex, 5 mM Mg^{2+}, 80–840 μM εCoA, and

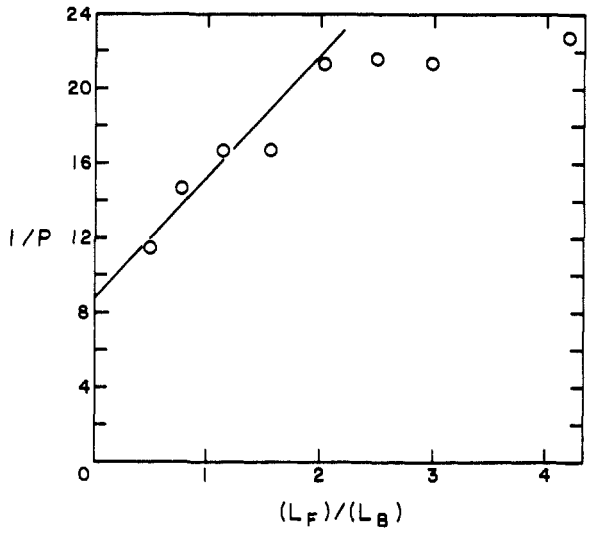

FIGURE 4: A plot of $1/P$ vs. $(L_F)/(L_B)$ where P is the measured steady-state polarization (305-nm excitation, 405-nm emission), (L_F) is the total free and nonspecifically bound ϵCoA concentration, and (L_B) is the specifically bound (CoA displaceable) ϵCoA concentration. The data were obtained by titration at 5 °C of 7.14 μM apo complex with 80–840 μM ϵCoA in 5 mM Mg^{2+}, 10 mM dithiothreitol, 0.02 M potassium phosphate (pH 7.0). The $(L_F)/(L_B)$ values were calculated from eq 4 with $n = 25.2$, $K_D = 57.8$. The line is a least-squares fit for the data obtained with $(L_F)/(L_B) < 2.5$ according to eq 5.

10 mM dithiothreitol). The results are summarized in Figure 4 as a plot of $1/P$ vs. $(L_F)/(L_B)$. The values of L_F and L_B were calculated from the binding data presented earlier. Extrapolation of $1/P$ to $(L_F)/(L_B) = 0$ gives a value of 0.115 for the polarization of specifically bound ϵCoA. (The corresponding anisotropy is 0.079.) The estimated limiting polarization of the ϵ-adenosine chromophore at 305-nm excitation is 0.300 (Secrist et al., 1972).

Using the values of the quantum yield of ϵCoA, the overlap integral for ϵCoA emission and FAD absorption, the refractive index of water, and $K^2 = \frac{2}{3}$, the value of R_0 for the ϵCoA–FAD donor–acceptor pair is 36.0 Å. The results obtained indicate that the *maximum* efficiency of energy transfer is 0.1; this efficiency gives a *minimum* value for the distance between the donor and acceptor of 51.9 Å. This is a lower bound, since less energy transfer and transfer to multiple acceptors would give a longer distance. Therefore, the distance between ϵCoA on E_2 and FAD on E_3 is estimated to be greater than 50 Å, assuming the value for K^2, which is based on both a freely rotating donor and acceptor, is not seriously in error.

Discussion

As in previous studies of ligand binding to the pyruvate dehydrogenase complex (Shepherd and Hammes, 1976), the data for ϵCoA binding to the enzyme complex are complicated by nonspecific binding; however, the following conclusions can be derived. The ϵCoA binds at approximately 22–25 sites which are CoA displaceable from E_2 either with the apo complex or with the E_2–E_3 subcomplex. The dissociation constant is 2–3 times larger for binding to the subcomplex. Both of the measured stoichiometries are within the experimental uncertainties of the number of E_2 subunits in the enzyme complex, 24 (Eley et al., 1972). No cooperativity is evident for either binding isotherm. The advantage gained by the reduction in the amount of nonspecific binding to the E_2–E_3 subcomplex relative to the intact complex unfortunately is offset by the weaker binding. Kinetically, ϵCoA is active as a substrate in the NADH assay and has its smallest Michaelis constant under the conditions used for the direct binding experiments. For a complex enzyme mechanism such as this, the apparent Michaelis constant has no simple meaning, but the measured equilibrium dissociation constant is significantly larger than the Michaelis constant determined under identical conditions (except for the absence of the other substrates for E_1 and E_3). The Michaelis constant for ϵCoA is consistently larger than that for CoA, while the opposite relationship is found for the maximal velocities associated with the two substrates.

The problems associated with the utilization of fluorescence energy transfer to measure distances in this particular system have been discussed previously (Moe et al., 1974; Shepherd and Hammes, 1976). In the present case the unchanged quantum yield of ϵCoA on binding to the enzyme reduces the sensitivity of detecting small changes, but clearly no more than 10% energy transfer is observed. The assumption that $K^2 = \frac{2}{3}$ is the major source of uncertainty in calculating the minimum distance between the donor and acceptor. In this particular case, the use of $\frac{2}{3}$ is not grossly in error since ϵCoA is definitely somewhat mobile: the polarization of bound ϵCoA is only 0.38 of the limiting polarization.

The minimum calculated value of R, subject to the above restrictions, is 52 Å. This is considerably larger than the 28 Å predicted by the proposed model of a single rotating lipoic acid interacting with all three catalytic sites. However, these results are consistent with the distances expected for the alternative formulation of the model employing lipoic acid interchanges. Thus, the fluorescence resonance energy transfer measurements made thus far indicate the catalytic sites of the three enzymes comprising the multienzyme complex are not close together. Further experiments are in progress to test this conclusion.

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The "Phosphoryl-Enzyme" from Phosphoglycerate Kinase†

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Appendix: Crystalline 3-Phospho-D-glycerate Kinase from Horse Muscle†

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ABSTRACT: The "phosphoryl-enzyme" prepared from phosphoglycerate kinase and adenosine 5′-triphosphate in the presence of an adenosine 5′-diphosphate trap is shown to contain stoichiometric amounts of 3-phosphoglycerate. This "phosphoryl-enzyme" is chemically competent, but is probably just a tight complex between 1,3-bisphosphoglycerate and the enzyme. The two partial exchange reactions (between adenosine 5′-diphosphate and adenosine 5′-triphosphate, and between 3-phosphoglycerate and 1,3-bisphosphoglycerate) can both be observed, but their rates are very much slower than the rate of overall catalysis. No substrate analogue was found that accelerated the partial exchange reactions. Catalysis of each of the two exchange reactions and of the kinase reaction coincides after isoelectric focusing of purified enzyme, but the amount of cosubstrate necessary to cause the observed partial exchange rates is so small that these reactions may well be artifactual. The balance of evidence does not support a ping-pong pathway *via* phosphoryl-enzyme, and the reaction may be a sequential one in which the phosphoryl group is transferred between substrates in a ternary complex. The results point to the dangers in the interpretation of experiments where very small amounts of contaminating cosubstrate can lead to large kinetic effects, and to the possibility of mistaken deductions about the identity of reaction intermediates.

Phosphoglycerate kinase (ATP-3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3), which catalyzes the transfer of a phosphoryl group between 1,3-bisphosphoglycerate and ADP, has been the subject of considerable mechanistic controversy. The elucidation of the mechanism is complicated by conflicting evidence from experiments using several different techniques. Steady-state kinetic data (Larsson-Raźniekiewicz, 1967; Larsson-Raźniekiewicz and Arvidsson, 1971; Janson and Cleland, 1974; Lee and O'Sullivan, 1975) indicate that the mechanism may be of the "random sequential" type. Evidence suggesting that the phosphoglycerate kinase catalyzed reaction proceeds *via* a pathway that involves a discrete, isolable, covalent phosphoryl-enzyme intermediate (Walsh and Spector, 1971; Roustan et al., 1973; Brevet et al., 1973) has also been reported. The chemical competence of the postulated phosphoryl-enzyme was demonstrated by the complete transfer of phosphate to ADP and to 3-phosphoglycerate (3-PGA)[1] (Walsh and Spector, 1971). The "U"-shaped pH–stability profile of the phosphoryl-enzyme and the observation that hydroxylaminolysis led to the facile release of phosphate (Walsh and Spector, 1971) suggested the presence of an acyl phosphate linkage. Indeed, Brevet et al. (1973) have proposed that the phosphoryl group is attached to the γ-carboxyl group of a glutamyl residue, on the basis of experiments in which the isolated phosphoryl-enzyme was treated with hydroxylamine and the product subjected to a Lossen rearrangement. Further evidence for the existence of a phosphoryl-enzyme has come from the reported partial isotopic exchange reaction between ATP and [^{3}H]ADP, apparently in the absence of the cosubstrate (Walsh and Spector, 1971; Roustan et al., 1973). The concept of "substrate synergism" (Bridger et al., 1968) was invoked, since this exchange reaction was markedly accelerated by low concentrations of 3-PGA.

Certain inconsistencies, however, caused us to reinvestigate the nature of this apparent phosphoryl-enzyme. For instance, the rate of the partial isotopic exchange reaction is very slow, the proportion of yeast enzyme that can be phosphorylated is capriciously variable, and the horse enzyme cannot be phosphorylated. Moreover, if the mechanism does involve a viable

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[1] Abbreviations used are: 3-PGA, 3-phospho-D-glycerate; BPGA, 1,3-bisphospho-D-glycerate; E ~ P, phosphoryl-enzyme; P_i, inorganic phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).