Fluorescence Energy Transfer Measurements between Ligand Binding Sites of the Pyruvate Dehydrogenase Multienzyme Complex†

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ABSTRACT: The interaction of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* with 8-anilino-1-naphthalenesulfonate (ANS), pyruvate, and acetyl-CoA has been investigated using equilibrium binding, steady-state fluorescence, and fluorescence lifetime measurements. The fluorescence of ANS is greatly enhanced when bound to the enzyme complex and to the pyruvate dehydrogenase component of the complex. Approximately 22 molecules of ANS are bound to a molecule of the complex with a binding constant of 3.69 μM in 0.02 M potassium phosphate (pH 7.0). Direct and competitive binding measurements indicate that about 42 pyruvate binding sites are present per mole of enzyme complex which has been stripped of thiamine diphosphate; the number of binding sites is reduced to 28.5 in the presence of a saturating concentration of thiochrome diphosphate, a thiamine diphosphate analogue. The dissociation constant for pyruvate to the enzyme complex in the presence of thiochrome diphosphate is 308 μM in 0.02 M potassium phosphate (pH 7.0). Pyruvate, thiochrome diphosphate, and acetyl-CoA all displace ANS from the enzyme complex. In the cases of pyruvate and thiochrome diphosphate, the concentration dependence of the displacements suggests the displacement is allosteric, while in the case of acetyl-CoA direct competition appears to be involved. GTP decreases the effect of acetyl-CoA on ANS binding. Direct binding studies of acetyl-CoA to the enzyme complex indicate that 24–26 bound acetyl-CoA molecules per complex can be readily displaced by ANS, and the binding of acetyl-CoA to these sites displays positive cooperativity. Fluorescence energy transfer measurements between bound ANS on the pyruvate dehydrogenase enzyme and FAD on the dihydrolipoyl dehydrogenase enzyme indicate, assuming the emission and absorption dipoles are randomly oriented, that these two probes must be at least 58 Å apart in the intact complex.

The pyruvate dehydrogenase multienzyme complex of *Escherichia coli* has been shown to be an arrangement of three enzymes which catalyze decarboxylation of pyruvate and formation of acetyl-CoA through the reactions shown in eq 1–5.

$$\text{pyruvate} + E_1[\text{-TPP}] \xrightarrow{Mg^{2+}} CO_2 + E_1[\text{hydroxyethyl-TPP}] \quad (1)$$

$$E_1[\text{hydroxyethyl-TPP}] + E_2[\text{Lip-S}_2] \rightleftharpoons E_1[\text{-TPP}] + E_2[\text{HS-Lip-}S\text{-acetyl}] \quad (2)$$

$$E_2[\text{HS-Lip-S-acetyl}] + \text{CoA} \rightleftharpoons E_2[\text{Lip-(SH)}_2] + \text{acetyl-CoA} \quad (3)$$

$$E_2[\text{Lip-(SH)}_2] + E_3[\text{FAD}] \rightleftharpoons E_2[\text{Lip-S}_2] + E_3[\text{FAD(red)}] \quad (4)$$

$$E_3[\text{FAD(red)}] + NAD^+ \rightleftharpoons E_3[\text{FAD}] + \text{NADH} + H^+ \quad (5)$$

The three enzymes are pyruvate dehydrogenase (E_1), dihydrolipoyl transacetylase (E_2), and dihydrolipoyl dehydrogenase (E_3) which utilize the cofactors thiamine diphosphate (TPP), FAD, and lipoic acid (Lip-S_2). The bracketed components are postulated tightly bound enzyme intermediates. This sequence requires the lipoic acid moiety to be able to react at binding and catalytic sites on all three enzymes (Koike and Reed, 1960). A mechanism has been proposed in which a single lipoic acid rotates between the sites of the three enzymes. Since lipoic acid is covalently attached to an ϵ-amino group of a lysine of E_2 and has an extension of about 14 Å, the three active sites must be situated within a sphere 28 Å in diameter if this mechanism is correct. The possibility of acetyl group transfer between lipoic acid arms also has been proposed which would extend the maximum distance between the catalytic sites to 56 Å (Koike et al., 1963).

The distance between thiochrome diphosphate, a fluorescent TPP analogue, on E_1 and the FAD on E_3 has been estimated to be 45 Å from fluorescence resonance energy transfer measurements (Moe and Hammes, 1974; Moe et al., 1974). Some uncertainty in this distance exists because of the very short fluorescence lifetime of thiochrome diphosphate and the uncertainty in the relative orientation of the transition dipoles of acceptor and donor molecules. The work presented here is a continuation of these specific site to site distance measurements using singlet–singlet fluorescence resonance energy transfer. The efficiency of energy transfer, E, from a fluorescent donor molecule to an absorbing acceptor molecule is defined by:

$$E = 1 - Q_{D\rightarrow A}/Q_D = 1 - \tau_{D\rightarrow A}/\tau_D \quad (6)$$

where Q and τ symbolize the quantum yield and fluorescence lifetime of the donor molecule, and the subscripts D $\rightarrow$ A and D refer to measurements made in the presence of the acceptor and in its absence, respectively. The relationship between the transfer efficiency and the distance, R, between a donor and acceptor for an isolated pair is given by:

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

In this equation R_0 is the distance of separation at which the transfer efficiency is 0.5 and is given by eq 8 (Förster, 1966; Stryer and Haugland, 1967):

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

Here n is the refractive index, K^2 is the orientation factor describing the mutual orientation of the donor emission dipole to the acceptor absorption dipole, and J is the integral of the spectral overlap between the normalized donor fluorescent emission spectrum and the acceptor absorption spectrum as given by:

$$J = \int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda / \int F(\lambda) d\lambda \quad (9)$$

In this equation F is the donor fluorescence, ϵ the molar extinction coefficient of the acceptor, and λ is the wavelength.

In this study, 8-anilino-1-naphthalenesulfonic acid (ANS),[1] which binds tightly and specifically to the acetyl-CoA site on the pyruvate dehydrogenase enzyme (E_1), was used as a fluorescent donor molecule, and the bound FAD on E_3 was used as the acceptor. The results indicate that these sites are at least 58 Å apart. The binding stoichiometry of pyruvate, acetyl-CoA, and ANS to the multienzyme complex, and the effect of other ligands and cofactors on ANS fluorescence and binding were also determined.

Materials and Methods

Chemicals. The ANS was obtained from Eastman Kodak, quinine bisulfate was from Aldrich, and all other biochemicals were from Sigma. The ANS was recrystallized four times from water as the Mg^{2+} salt. Thiochrome diphosphate was prepared as previously described (Moe and Hammes, 1974). The [2-^{14}C]pyruvic acid (Amersham-Searle 8.54 Ci/mol) was purified by preparative paper chromatography in ethanol–methanol–water (4.5:4.5:1) (Neish, 1957). The concentration of the pyruvate stock solution was determined using the lactic dehydrogenase assay (Meister, 1950), and the stock solution was stored at −10°. The [1-^{14}C]acetyl-CoA (Amersham-Searle 46.8 Ci/mol) was used directly. Analytical thin layer cellulose chromatography with ethanol–1 M ammonium acetate (50:20) gave a radiochemical purity of 99%. The concentration of the stock solution was determined by ultraviolet absorption (Stadtman, 1957), and the stock solution was stored at −10°. All other chemicals were the best commercial grades, and deionized, distilled water was used in all solutions.

Pyruvate Dehydrogenase Complex. The pyruvate dehydrogenase complex was prepared from the frozen *E. coli* cell paste (Strain B late-log harvest, Miles Labs.) according to the method of Reed and Willms (1966) using an ammonium sulfate precipitation to separate the pyruvate dehydrogenase complex from the α-ketoglutarate complex. The thiamine diphosphate was removed from the enzyme by dialysis against 0.1 M potassium phosphate (pH 8.25) and 2 mM EDTA at 4° followed by extensive dialysis against 0.02 M potassium phosphate (pH 7.0). Not all of the thiamine diphosphate is removed by this method since some residual enzyme activity is obtained without the addition of thiamine diphosphate to the assay mixture. Hereafter the complex with at least 99% of thiamine diphosphate removed will be referred to as an apo complex.

Flavine was removed from the complex at pH 3.6 using a procedure similar to that previously described (Koike and Reed, 1960). Approximately 65–75% of the FAD can be removed without altering the specific activity as measured by the ferricyanide assay. The specific activity, measured by the NADH assay, is proportional to the amount of FAD present in the complex. The amount of FAD present was determined spectrophotometrically using an extinction coefficient of 11 300 M^{-1} cm^{-1} at 450 nm (Whitby, 1953).

The activity of the apoenzyme complex was measured by the formation of NADH in 0.1 M potassium phosphate (pH 8.0) at 30°, and the activity of the pyruvate dehydrogenase component was measured by the ferricyanide reduction assay in 0.05 M tricine (pH 7.5) at 30° (Schwartz et al., 1968). Protein concentrations were determined by the method of Lowry et al. (1951). The specific activity of the complex varied from 24 to 32 (μmol of NADH formed per min per mg of protein). The enzyme complex concentration was calculated assuming a molecular weight of 4.6×10^6 (Eley et al., 1972).

The pyruvate dehydrogenase enzyme and the subcomplex of dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase were obtained by resolution of the enzyme complex on Sepharose 6B at pH 9.0 in a tricine–ethanolamine buffer (0.02 M) following essentially the same procedure described elsewhere for resolution of the α-ketoglutarate dehydrogenase complex from *E. coli* (Pettit et al., 1973).

Binding Measurements. The binding of [2-^{14}C]pyruvate to the apopyruvate dehydrogenase complex was studied using the forced dialysis method at 4° (Cantley and Hammes, 1973). Measurements were completed within 30 min to minimize pyruvate decarboxylation (less than 5% at 4°) caused by residual thiamine diphosphate or covalent labeling of the complex. To prevent protein from being forced through the membrane, PM 30 ultrafiltration membranes (Amicon) were used. These membranes must be used damp and contribute approximately a 3-μl dilution on initially forcing solution through the membrane. Therefore, three 12–15-μl samples were collected, and the last two samples were used to determine the concentration of free ligand. A total volume of about 230 μl was used, and the total ligand concentration was determined from an aliquot of the original solution. The concentration of apoenzyme complex was either 1.15 or 2.30 μM; the [2-^{14}C]pyruvate concentration varied from 10 to 1000 μM. A Beckman LS-255 liquid scintillation counter and a calibration curve were used to measure the radioactive decay rate of 10-μl samples in 10 ml of dioxane base scintillation fluid (Bray, 1960).

The binding of [1-^{14}C]acetyl-CoA to the apopyruvate dehydrogenase complex and the subcomplex of E_2–E_3 was studied in a similar manner. Measurements were completed within 60 min to minimize acetyl-CoA decomposition and possible acetylation of the enzyme complex. The concentration of the apoenzyme complex was varied from 1.70 to 3.52 μM; the concentration of [1-^{14}C]acetyl-CoA varied from 8 to 520 μM; and the concentration of E_2–E_3 was 1.60 or 3.20 μM.

Gel filtration of the apo complex in the presence of [2-^{14}C]pyruvate or [1-^{14}C]acetyl-CoA was done on a 1.8 cm diameter × 27 cm high column of Sephadex G-25 (Pharmacia) equilibrated with 0.02 M potassium phosphate (pH

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; Lip-S_2, lipoic acid; TPP, thiamine diphosphate; E_1, pyruvate dehydrogenase; E_2, dihydrolipoyl transacetylase; E_3, dihydrolipoyl dehydrogenase; E_2–E_3, pyruvate dehydrogenase subcomplex with E_1 removed; EDTA, ethylenediaminetetraacetic acid; tricine, *N*-tris(hydroxymethyl)methylglycine.

7.0) at 4°. The radioactivity and the 280- and 260-nm absorbances of the effluent were measured.

The stoichiometry for the binding of ANS to the apoenzyme complex was determined by the method of continuous variation (Job, 1928; Asmus, 1961). The sum of the concentrations of enzyme sites (assuming 24 sites per enzyme complex) and ANS was held at a constant value of 20 μM. The relative concentrations were varied from a ratio of 1:19 to 19:1, and ANS fluorescence at 465 nm (excitation at 350 nm) was used as the measure of the binding.

Spectroscopic Measurements. Ultraviolet and visible absorption measurements were made with a Zeiss PMQ II or a Cary 14 spectrophotometer. Steady-state fluorescence measurements were made with a Hitachi-Perkin-Elmer MPF-3 fluorescence spectrophotometer. For fluorescence measurements square (1 cm × 1 cm or 0.4 cm × 0.4 cm) or triangular (1 cm × 1 cm × 1.4 cm) 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 350 nm at 23° (Melhuish, 1962; Chen, 1967). The absolute quantum yield of quinine bisulfate was taken as 0.70 (Scott et al., 1970). Polarization and anisotropy measurements were made on enzyme-bound ANS. The values obtained were used to calculate the quantum yield of bound ANS from the corrected fluorescence spectrum using the relationship in eq 10 (Shinitzky, 1972):

$$I = I_0(1 + \tfrac{1}{4}r_a(3\cos^2\theta - 1)) \qquad (10)$$

where I is the measured intensity, I_0 is the true intensity, r_a is the steady-state anisotropy, and θ is the angle between excitation and viewed emission (i.e., 90°).

Fluorescence Lifetime Measurements. The ORTEC 9200 single photon nanosecond fluorescence system was used for fluorescence lifetime measurements as described elsewhere (Matsumoto and Hammes, 1975). Microcells of 0.4 cm × 0.4 cm were thermostated at 8° with dry N_2 circulated through the sample chamber. An interference filter (Ditric) with maximum transmittance at 470 nm and bandpass of 7 nm was used in the emission light path. The excitation wavelength was 350 nm as fixed by a monochromator with a bandwidth of 10 nm. All nanosecond decay spectra were accumulated for the same analysis time with conditions such that the dead time of the multichannel analyzer was zero. Scattering spectra of the enzyme (100 or 30% FAD content) alone were accumulated for the same time span. This spectrum, when subtracted from the corresponding spectrum containing enzyme and ANS, corrects for both the scattering contribution of the enzyme and the uniformly distributed random coincidences in the photon counting system. The fluorescence lifetime (τ) and the intensity factor (A) were found by employing a method of moments deconvolution for a single exponential decay (Yguerabide, 1972). A weighted sum of deviations squared was used in minimizing the fitting error by altering A and τ subject to the constraint that the total fluorescence intensity remains constant (eq 11). The value of $A\tau$ was altered to optimize the fit. This procedure also gives an estimate of the range in τ consistent with the data.

$$A\tau = \int_0^\infty Ae^{-t/\tau}\,dt \qquad (11)$$

The anisotropy decay of the bound ANS in the enzyme complex at 8° was studied using fluorescence depolarization (Yguerabide, 1972). The sample was excited at 340 nm and the emission was observed at 470 nm.

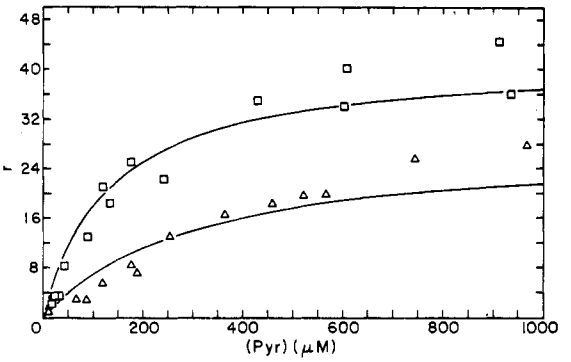

FIGURE 1: A plot of r vs. (Pyr), where r is the number of moles of [2-^{14}C]pyruvate bound per mole of apopyruvate dehydrogenase complex and (Pyr) is the concentration of free [2-^{14}C]pyruvate. The data were obtained by forced dialysis at 4°: (□) 1.15 or 2.30 μM apo complex and 10–1000 μM [2-^{14}C]pyruvate, (△) 1.15 or 2.30 μM apo complex, 2 mM thiochrome diphosphate, and 10–1000 μM [2-^{14}C]pyruvate. All solutions contained 2.2 mM $MgCl_2$ and 0.02 M potassium phosphate (pH 7.0). The lines were calculated with eq 12 using the parameters given in the text.

Results

The results of the measurements of pyruvate binding to the apopyruvate dehydrogenase complex and to the apo complex in the presence of 2 mM thiochrome diphosphate in 0.02 M potassium phosphate (pH 7.0) and 2.2 mM Mg^{2+} at 4° are summarized in Figure 1 as plots of r, the moles of ligand bound per mole of enzyme, vs. the free pyruvate concentration. The concentration of thiochrome diphosphate, a competitive inhibitor of TPP, was sufficient to essentially saturate the TPP binding sites.

The data were fit to eq 12 which assumes a single class of binding sites:

$$r = n_1(\text{Pyr})/[K_1 + (\text{Pyr})] \qquad (12)$$

In eq 12, n_1 is the number of independent binding sites with intrinsic dissociation constant K_1 and (Pyr) is the concentration of free pyruvate. For the apoenzyme complex, a least-squares fitting of the data gives n_1 = 42 (±3) and K_1 = 133 (±3) μM. For pyruvate binding in the presence of thiochrome diphosphate, n_1 = 28.5 (±3) and K_1 = 308 (±6) μM. The lines in Figure 1 were calculated using these parameters and eq 12. The reversibility of the binding process was checked by passage of a mixture of [2-^{14}C]pyruvate (582 μM) and apo complex (3.75 μM) in 1 mM Mg^{2+}, 0.02 M potassium phosphate (pH 7.0), 4° through a G-25 Sephadex column. No incorporation of radioactivity into the enzyme occurred with a 30-min incubation period before elution.

The results of the measurement of acetyl-CoA binding to the apopyruvate dehydrogenase complex and to the apo complex in the presence of 0.75 or 1.5 mM ANS in 0.02 M potassium phosphate (pH 7.0) and 2 mM Mg^{2+} at 4° are summarized in Figure 2 as plots of r vs. the free acetyl-CoA concentration. A binding isotherm for acetyl-CoA binding to the ANS binding sites was constructed from the data in Figure 2 by plotting Δr vs. free acetyl-CoA concentration (L) (Figure 3A) where Δr is defined as the difference in r values between acetyl-CoA binding with and without a saturating concentration of ANS at the same total acetyl-CoA and apo complex concentrations. These data are also shown

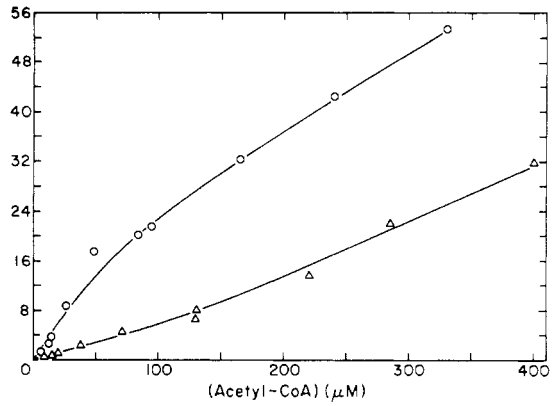

FIGURE 2: A plot of r vs. (acetyl-CoA), where r is the number of moles of [1-^{14}C]acetyl-CoA bound per mole of apopyruvate dehydrogenase complex and (acetyl-CoA) is the concentration of free [1-^{14}C]acetyl-CoA. The data were obtained by forced dialysis at 4°: (○) 1.70–3.52 μM apo complex and 8–520 μM [1-^{14}C]acetyl-CoA, (△) 1.70–3.52 μM apo complex, 0.75 (acetyl-CoA < 160 μM) or 1.5 mM ANS (acetyl-CoA > 160 μM), and 8–520 μM [1-^{14}C]acetyl-CoA. All solutions contained 2.2 mM $MgCl_2$ and 0.02 M potassium phosphate (pH 7.0). The lines have no theoretical significance.

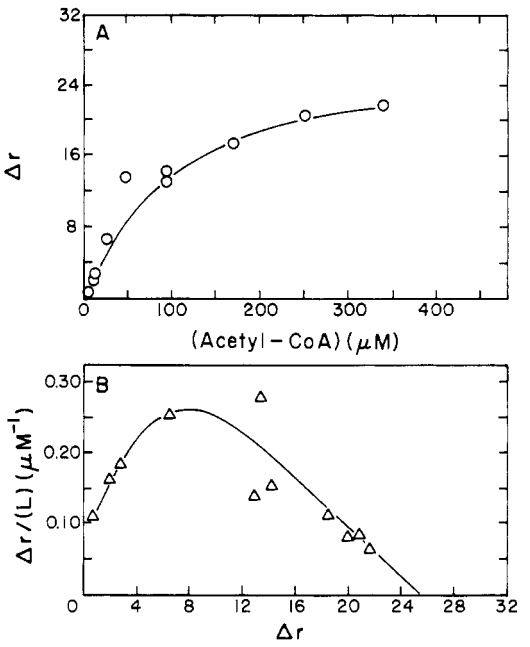

FIGURE 3: (A) A plot of Δr vs. (acetyl-CoA), where Δr is the number of moles of acetyl-CoA bound per mole of apo complex that are displaceable by saturating ANS (0.75 or 1.5 mM) and (acetyl-CoA) is the concentration of free [1-^{14}C]acetyl-CoA found in the absence of ANS. The data are derived from Figure 2. (B) A plot of $\Delta r/(L)$ vs. Δr where (L) is the concentration of free [1-^{14}C]acetyl-CoA. The data are derived from Figure 3A. The lines have no theoretical significance.

in Figure 3B as a plot of $\Delta r/(L)$ vs. Δr (Scatchard, 1949); the maximum observed indicates positive cooperativity in the acetyl-CoA binding to the ANS displaceable sites. The data can be extrapolated to a total of 24–26 ANS displaceable sites. Acetyl-CoA also binds to the isolated E_2–E_3 complex appreciably, but this binding only decreases 10–15% (at r = 15) in the presence of 1.5 mM ANS. The passage of a mixture of [1-^{14}C]acetyl-CoA and the apo complex (1.42 or 2.38 μM apo complex, 2.0 mM Mg^{2+}, and 39.5 or 615 μM acetyl-CoA, in 0.02 M potassium phosphate (pH 7.0)) through a G-25 Sephadex column indicated that radioactive label was not covalently incorporated into the enzyme complex within the incubation time of 15 min.

The apoenzyme complex (0.0075 μM) was titrated with ANS in 0.02 M potassium phosphate (pH 7.0) and 1 mM Mg^{2+} at 8° by following the increase in fluorescence at 465 nm with an excitation wavelength of 350 nm. The results obtained are presented in Figure 4A as a plot of the relative ANS fluorescence vs. the total concentration of ANS. If a single class of binding sites with an intrinsic dissociation constant, K_D, is assumed and if the fluorescence change is proportional to r, the reciprocal of the fluorescence change, $1/F$, is given by:

$$\frac{1}{F} = \frac{1}{f(E_0)} + \frac{1}{f(E_0)}\left[\frac{K_D}{(ANS)}\right] \qquad (13)$$

In this equation f is the proportionality constant relating the fluorescence to the concentration of bound ANS and (E_0) is the total enzyme concentration. In deriving eq 13 the assumptions also have been made that only bound ANS fluoresces and that the concentration of ANS is much greater than that of the enzyme. Both of these assumptions are valid under the experimental conditions employed. A plot of $1/F$ vs. $1/(ANS)$ is shown in Figure 4B, and a weighted least-squares analysis of the data gives a K_D of 3.69 μM. The value of K_D was identical in 0.1 M tricine (pH 8.0) and 0.1 M potassium phosphate (pH 8.0), but was found to be 13.3 μM in 0.02 M tricine (pH 7.0). The magnitude of the fluorescence change was the same in all cases. When the separated pyruvate dehydrogenase enzyme (E_1) was titrated with ANS at 8° in 0.02 M potassium phosphate (pH 7.0) containing 0.090 μM E_1 dimer, K_D was found to be 3.88 μM, and the change in fluorescence was the same as observed with the total complex. Under the same conditions titration of 0.0074 μM E_2–E_3 subcomplex (2.22×10^6 molecular weight assumed; Eley et al., 1972) gave a K_D of 17.9 μM with a maximum fluorescence intensity 0.28 of that for the intact complex or E_1 alone.

As shown in Figure 5A, addition of both pyruvate and thiamine diphosphate markedly decreases the fluorescence of the bound ANS. The concentrations at which the fluorescence of bound ANS is one-half its original value are 400 and 500 μM for pyruvate and thiamine diphosphate, respectively, at an ANS concentration of 7.32 μM and an apoenzyme concentration of 0.0139 μM in 0.02 M potassium phosphate (pH 7.0) at 8°. Under identical experimental conditions, if thiamine diphosphate and pyruvate are added to the bound ANS apocomplex in a ratio of 12:65, an even greater decrease in fluorescence occurs (Figure 5A). (The ratio of thiamine diphosphate to pyruvate was based on their apparent Michaelis constants, 12 and 65 μM, respectively, as determined from steady-state kinetics at 30° (Moe and Hammes, 1974; Schwartz and Reed, 1970).) Simultaneous addition of 12 μM thiamine diphosphate and 65 μM pyruvate results in more than a 50% decrease in fluorescence; all but 14% of the ANS fluorescence can be removed by simultaneous addition of these ligands.

Under similar conditions (0.0134 μM apoenzyme in 0.02 M potassium phosphate (pH 7.0), 7.43 μM ANS, 8°) the effect of known effectors of the enzymatic reaction can be observed. In Figure 5B GTP is shown to cause a small increase in ANS fluorescence. The addition of acetyl-CoA causes a large decrease in fluorescence. The concentration of acetyl-CoA at which the ANS fluorescence is half the original value is 15 μM. The addition of GTP to ANS

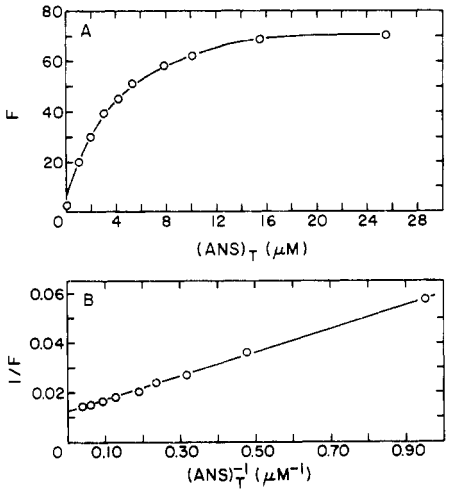

FIGURE 4: (A) A plot of F vs. $(ANS)_T$ where F is the change in ANS fluorescence (350-nm excitation, 465-nm emission) and $(ANS)_T$ is the concentration of ANS. The data were obtained by fluorescence titration at 8° with 0.0075 μM apo complex and 1–30 μM ANS in 2.2 mM $MgCl_2$ and 0.02 M potassium phosphate (pH 7.0). (B) A plot of $1/F$ vs. $(ANS)_T^{-1}$. The data were derived from Figure 4A. The line is a least-squares fit using eq 13.

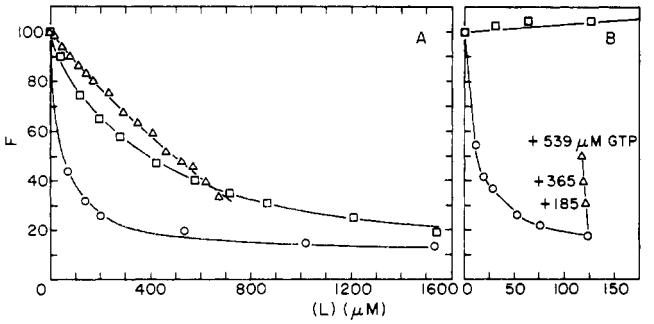

FIGURE 5: (A) A plot of F vs. (L) where F is the relative fluorescence of ANS (350-nm excitation, 465-nm emission) and (L) is the total concentration of added ligand. The data were obtained by fluorescence measurements at 8°: (□) 50–1550 μM pyruvate, (△) 12–670 μM TPP, and (○) 65–1530 μM pyruvate with TPP simultaneously added in a ratio of 12:65 TPP to pyruvate. The concentration of apo complex was 0.0139 μM with 7.32 μM ANS. (B) A plot of F vs. (L) where F and (L) are as in Figure 5A. The data were obtained by fluorescence measurements at 8°: (□) 32–125 μM GTP, (○) 10–125 μM acetyl-CoA, (△) 125 μM acetyl-CoA with 185–539 μM GTP added in the designated increments. The concentration of apo complex was 0.0134 μM with 7.43 μM ANS. For both Figure 5A and 5B all solutions contained 2.2 mM $MgCl_2$ and 0.02 M potassium phosphate (pH 7.0). The lines are of no theoretical significance.

bound apoenzyme in the presence of 125 μM acetyl-CoA results in the increase in ANS fluorescence shown in Figure 5B.

Because ANS fluorescence is extremely sensitive to changes in the local environment, the changes in fluorescence caused by the binding of other ligands are not necessarily directly related to an enhancement or decrease in ANS binding. Direct binding measurements of [2-^{14}C] pyruvate to the apoenzyme complex in the presence of ANS indicated a maximum decrease of 50% in the extent of pyruvate binding. Pyruvate (3.5 mM) decreases acetyl-CoA binding from $r = 10$ to $r = 2$.

The stoichiometry of ANS binding to the apoenzyme complex was determined by the method of continuous variation by plotting the fluorescence at 465 nm vs. the mole fraction of ANS relative to the sum of the concentrations of ANS and apoenzyme. The fluorescence was corrected for light scattering and absorbance of exciting light and emitted fluorescence although the corrections were less than 10%. The maximum fluorescence change occurs at an ANS mole fraction of 0.475, which corresponds to 22 ANS binding sites per enzyme complex.

The absorption spectrum of ANS changes slightly when it binds to the apoenzyme, and a difference spectrum between free and bound ANS in 0.02 M potassium phosphate (pH 7.0) at 8° is shown in Figure 6A where the change in molar extinction coefficient is plotted vs. the wavelength. The concentration of ANS was 78 μM and the concentration of apo complex was 0.540 μM. The calculated ratio of bound ANS to apoenzyme is 22.7. The largest spectral change occurs at 390 nm where a 120% change in the molar extinction occurs. A reference spectrum of ANS in 0.02 M potassium phosphate (pH 7.0) at 23° is included in Figure 6A. The extinction coefficient at 350 nm was taken as 4950 M^{-1} cm^{-1} (Weber and Young, 1964).

The polarization of bound ANS as measured by steady-state fluorescence in 0.02 M potassium phosphate (pH 7.0), 0.014 μM apoenzyme complex, 84.5 μM ANS, and 1.0 mM Mg^{2+} at 4° with an excitation wavelength of 350 nm and an emission wavelength of 465 nm is 0.200 (±0.005). The corresponding anisotropy is 0.146. The polarization of ANS bound to apoenzyme with only 30% of the normal amount of FAD was within a few percent of this value. The polarization of the bound FAD on the apoenzyme complex under the same experimental conditions was found to be 0.472

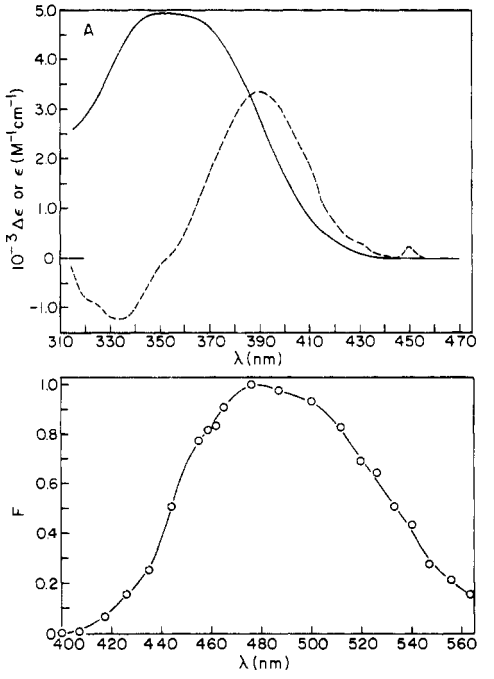

FIGURE 6: (A) A plot of $\Delta\epsilon$ or ϵ vs. wavelength for ANS where $\Delta\epsilon$ is the change in molar extinction coefficient when ANS is bound to the apo complex. The difference spectrum (- - -) was obtained at 8° with 78.7 μM ANS and 0.540 μM apo complex using 0.44-cm path length cells, and the absorption spectrum of ANS (23 μM) was obtained at 23° (—). The buffer used for both contained 2.2 mM $MgCl_2$ and 0.02 M potassium phosphate (pH 7.0). (B) A plot of F vs. wavelength where F is the corrected and normalized emission spectrum of ANS bound to the apo complex (350-nm excitation) at 8°. The data were obtained by comparison to an emission spectrum of quinine bisulfate (0.59 μM) in 0.1 N H_2SO_4 at 23° under identical instrumental conditions. The solution contained 9.48 μM ANS, 0.0136 μM apo complex, 2.2 mM $MgCl_2$, and 0.02 M potassium phosphate (pH 7.0).

with an excitation wavelength of 460 nm and an emission wavelength of 540 nm. The corresponding anisotropy is 0.374. The fluorescence depolarization of ANS bound to the apo complex showed a rotational correlation time of greater than 400 nsec indicating the ANS is quite immobile.

The quantum yield of bound ANS (0.02 *M* potassium phosphate (pH 7.0), 9.41 μM ANS, 0.014 μM apoenzyme complex, $r \sim 17.0$) with 30% FAD content was determined to be 0.77 ± 0.04 by steady-state fluorescence measurements at 4°. When eq 10 is used to correct for the anisotropy of bound ANS, the quantum yield is 0.80. The quantum yield of ANS bound to the apoenzyme complex with 100% FAD content under similar conditions was 0.80 (±0.05) which, when corrected for anisotropy, gives a quantum yield of 0.83. Thus within the experimental uncertainties of the quantum yield measurements, energy transfer between bound ANS and FAD is not observed.

Fluorescence lifetime measurements of bound ANS were carried out in 0.02 *M* tricine or potassium phosphate (pH 7.0) at 8°. The apoenzyme concentration was 1.19 μM and the total ANS concentration was 167 μM which corresponds to $r \sim 23.5$. The lifetime of ANS bound to the apoenzyme complex with 100% FAD content ranged from 18.1 to 18.3 nsec; with 30% of the FAD bound, the lifetime ranged from 17.9 to 18.1 nsec. The estimated uncertainty in these lifetimes is about ±5%. Thus within the experimental uncertainties, the fluorescence lifetime is independent of the FAD content of the enzyme complex.

Using the value of the ANS quantum yield, the corrected emission spectrum of ANS bound to apoenzyme complex with 30% FAD content (Figure 6B), the absorption spectrum of bound FAD (Moe et al., 1974), and assuming $K^2 = \frac{2}{3}$, R_0 was calculated to be 39.9 Å (eq 7). This calculation assumes each bound ANS is a donor to a single FAD; thus a minimum value of R_0 is obtained. The *maximum* efficiency of energy transfer between bound ANS and FAD which is consistent with the fluorescence quantum yield and lifetime measurements is 0.1. If the bound ANS molecules are equidistant from the bound FAD molecules, the minimum distance between them is 58 Å (eq 7).

Discussion

The binding data are complicated by nonspecific binding of ligands to the enzyme, but the following conclusions can be derived from the data presented. Pyruvate binds at approximately 28 sites in the presence of a saturating concentration of thiochrome diphosphate. In the absence of thiochrome diphosphate, the number of apparent binding sites increases; whether this is due to direct inhibition by thiochrome diphosphate or to a conformational change cannot be ascertained. ANS binds to approximately 22 sites on E_1. Both of these stoichiometries are within the experimental uncertainties of the number of E_1 subunits present in the enzyme complex, 24 (Eley et al., 1972). No evidence of cooperativity is observed in the binding isotherms for these ligands. Acetyl-CoA binds to a large number of sites on the enzyme complex, but only 24–26 of these sites also bind ANS. These results, together with those obtained with the separated E_1 and E_2–E_3 enzymes, indicate that ANS and acetyl-CoA are very probably binding to the same sites on E_1. While pyruvate and TPP clearly influence the binding of acetyl-CoA and ANS, direct inhibition appears not to be involved so that acetyl-CoA apparently binds at a site distinct from the catalytic site, although allosteric interactions between the two types of sites occur. The binding of acetyl-CoA to this allosteric site apparently involves positive cooperativity. However, the data are insufficient to develop a detailed binding model. The quenching of ANS fluorescence by pyruvate and TPP is greater than predicted from direct binding measurements suggesting that a conformational change occurs; presumably acetyl-CoA binding also would involve a conformational change. Kinetic studies have suggested acetyl-CoA is a competitive inhibitor of pyruvate binding and that GTP relieves this inhibition (Schwartz and Reed, 1970; Schwartz et al., 1968). GTP also relieves the acetyl-CoA inhibition of ANS fluorescence.

The application of fluorescence energy transfer to measuring distances in biological systems requires that any fluorescence quenching observed not be due to inner filter effects, environmental changes caused by introducing the acceptor molecule into the system, or displacement of the donor in the case of a noncovalent probe. In addition the location of the probes must be known. In the present case, ANS appears to bind specifically at the acetyl-CoA site on E_1, and FAD is known to be at the active site of E_3. The emission spectrum and the measured binding constants are essentially the same for the enzyme complex in the presence and absence of flavine and for the isolated E_1 species. This suggests environmental changes are not influencing the results.

Energy transfer clearly is not observed between ANS and FAD. Even assuming a generous experimental uncertainty of ±10%, the ANS and FAD sites must be greater than 58 Å apart. Previous results have indicated that the TPP site is approximately 45 Å from the bound FAD. Thus all available data indicate that E_1 and E_3 are quite far apart. Therefore, the proposed mechanisms of a single rotating lipoic acid molecule interacting with all three catalytic sites seems improbable. In calculating R_0, the assumption was made that $K^2 = \frac{2}{3}$. It is possible, but not very probable, that this could introduce a serious error in the estimate of the minimum distance between bound ANS and FAD. The bound ANS fluorescence is strongly polarized. (The polarization observed is 0.75 of the limiting polarization, and the rotational correlation time is quite long.) As long as K^2 is significantly different from zero, the calculated minimum distance cannot be seriously in error since the calculated distance is proportional to $(K^2)^{1/6}$. The state of E_1, as judged by the ferricyanide assay, is not significantly altered by removal of FAD so that it is unlikely the fluorescence of ANS is changed by removal of FAD per se.

In comparing the results presented here with those obtained from other laboratories (e.g., Speckhard and Frey, 1975; Schwartz et al., 1968), it should be noted that the activity of the enzyme (and presumably its other properties) is quite sensitive to pH, ionic strength, and specific buffers. The different bacterial sources used for the enzyme also might cause some differences in the properties of the enzyme. Positive cooperativity in the interaction of pyruvate with the enzyme complex and negative cooperativity for the acetyl-CoA interaction has been inferred from indirect measurements under somewhat different experimental conditions (Bisswanger and Henning, 1971), but within the experimental uncertainties, cooperativity is not observed in the pyruvate binding studies reported here.

Further experiments are in progress to ascertain the distances between the catalytic sites and other specific ligand binding sites of the pyruvate dehydrogenase enzyme complex.

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The Thermodynamics of the Self-Association of the Reduced and Carboxymethylated Form of ApoA-II from the Human High Density Lipoprotein Complex†

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ABSTRACT: The thermodynamics of the self-association of the reduced carboxymethylated form of apoA-II protein (molecular weight 8690) from human serum high density lipoproteins has been obtained at neutral pH by ellipticity measurements. Changes in secondary and tertiary structure accompany the association. The association is endothermal at low and exothermal at high temperatures, and involves a decrease in heat capacity of 1250 cal/(mol deg) at 25°.

In order to characterize more fully the molecular organization of lipoprotein particles we have undertaken a study of the molecular properties of the isolated components of the high density lipoprotein complex. In two previous publications of this series the molecular properties of human apoA-II and reduced and carboxymethylated apoA-II, i.e., Cm apoA-II, were reported (Gwynne et al., 1975; Osborne, et al., 1975). Human apoA-II, a disulfide dimer of two identical chains of 77 residues (Brewer et al., 1972), was shown to self-associate to a dimer in aqueous solution (mol wt 17380 → 34760) (Gwynne et al., 1975). The single chain, Cm apoA-II (mol wt 8690), also self-associates in aqueous solution to form a dimer of molecular weight 17380 (Osborne et al., 1975). The dimer form of both molecules has appreciable secondary and tertiary structure, whereas the monomers possess little organized structure and resemble random coils.

The thermodynamics of these self-associations are quite unusual since increasing temperature first increases and then decreases the association constant of Cm apoA-II. The association of the unreduced, native molecule, apoA-II,

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