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Fluorescent Derivatives of the Pyruvate Dehydrogenase Component of the *Escherichia coli* Pyruvate Dehydrogenase Complex†

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ABSTRACT: One sulfhydryl group per polypeptide chain of the pyruvate dehydrogenase component of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* was selectively labeled with *N*-[*p*-(2-benzoxazolyl)phenyl]-maleimide (NBM), 4-dimethylamino-4′-maleimidostilbene (NSM), and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) in 0.05 M potassium phosphate (pH 7). Modification of the sulfhydryl group did not alter the enzymatic activity or the binding of 8-anilino-1-naphthalenesulfonate (ANS) or thiochrome diphosphate to the enzyme. The fluorescence of the NBM or NSM coupled to the sulfhydryl group on the enzyme was quenched by binding to the enzyme of the substrate pyruvate the coenzyme thiamine diphosphate, the coenzyme analogue thiochrome diphosphate, the regulatory ligands acetyl-CoA, GTP, and phosphoenolpyruvate, and the acetyl-CoA analogue, ANS. Fluorescence energy transfer measurements were carried out for the enzyme-bound donor-acceptor pairs NBM-ANS, NBM-thiochrome diphosphate, ANS-DDPM, and thiochrome diphosphate-DDPM. The results indicate that the modified sulfhydryl group is more than 40 Å from the active site and ~49 Å from the acetyl-CoA regulatory site. Thus, a conformational change must accompany the binding of ligands to the regulatory and catalytic sites. Anisotropy depolarization measurements with ANS bound on the isolated pyruvate dehydrogenase in 0.05 M potassium phosphate (pH 7.0) suggest that under these conditions the enzyme is dimeric.

The *Escherichia coli* pyruvate dehydrogenase complex which catalyzes the overall reaction

$$CH_3COCOOH + CoA + DPN^+ \rightarrow \text{Acetyl-CoA} + CO_2 + DPNH + H^+ \quad (1)$$

has been separated into three component enzymes (Koike et al., 1963; Eley et al., 1972). The pyruvate dehydrogenase component, E_1,[1] which utilizes thiamine diphosphate as a co-

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[1] Abbreviations used are: E_1, pyruvate dehydrogenase; ANS, 8-anilino-1-naphthalenesulfonate; NBM, *N*-[*p*-(2-benzoxazolyl)phenyl]-maleimide; NSM, 4-dimethylamino-4′-maleimidostilbene; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; E_1-NBM, E_1-NSM, and E_1-DDPM, pyruvate dehydrogenase with a sulfhydryl group modified with NBM, NSM, and DDPM, respectively; CoA, coenzyme A; DPN, diphosphopyridine nucleotide.

factor has been shown to contain regulatory binding sites for phosphoenolpyruvate, GTP, and acetyl-CoA and the catalytic binding site for pyruvate (Schwartz et al., 1968; Schwartz and Reed, 1970a). Sedimentation equilibrium experiments have shown that in 0.05 M potassium phosphate (pH 7.0) E_1 has a molecular weight of 192 000. This, combined with the results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, indicates that the enzyme is a dimer at pH 7.0 (Eley et al., 1972).

Reaction of E_1 with organic mercurials indicated that a maximum of four sulfhydryl groups per protein of molecular weight 192 000 can be modified at pH 7.0. When the reaction was done in the absence of thiamine diphosphate and Mg^{2+}, two sulfhydryl groups per E_1 dimer reacted with a complete loss of the enzymatic activity. However, in the presence of thiamine diphosphate and Mg^{2+}, modification of the first two sulfhydryl groups did not alter the activity; further treatment with the mercurials inactivated the enzyme and resulted in the modification of two additional sulfhydryl groups. The sedimentation characteristics of the enzyme as well as its ability to reconstitute the pyruvate dehydrogenase complex by combination with the other two enzymes were not affected when inactivated by the mercurials (Schwartz and Reed, 1970b).

The work presented here indicates that two sulfhydryl groups per E_1 dimer can be modified with maleimide derivatives without altering the enzymatic activity. Fluorescence singlet–singlet energy transfer measurements suggest the sulfhydryl group on the E_1 chain is more than 40 Å away from the acetyl-CoA and the thiamine diphosphate binding sites. However, binding to any of the regulatory or catalytic sites results in quenching of the fluorescence of the probes on the sulfhydryl group, indicating a conformational change accompanies ligand binding to these sites.

Experimental Procedures

Materials. The NBM and NSM, purchased from Eastman Kodak, were used without further purification. The ANS, also from Eastman Kodak, was recrystallized four times from water as the Mg^{2+} salt. The concentration of the ANS solutions was measured spectrophotometrically using an extinction coefficient of 5100 M^{-1} cm^{-1} at 350 nm (as determined from dry weight). Quinine bisulfate and DDPM were obtained from Aldrich Chemical Co., and all other biochemicals from Sigma. Thiochrome diphosphate was prepared as previously described (Moe and Hammes, 1974), and an extinction coefficient of 2.06×10^4 M^{-1} cm^{-1} at 367 nm was used to determine its concentration. All other chemicals were the best available commercial grades, and deionized distilled water was used in all solutions. Unless otherwise noted, all measurements and preparations were done in 0.05 M potassium phosphate (pH 7.0) at 5 °C.

Preparation of Enzyme and Enzyme Derivatives. The preparation, activity measurements, and protein analyses of the pyruvate dehydrogenase enzyme complex and the pyruvate dehydrogenase component, E_1, were by standard procedures (Reed and Willms, 1966; Schwartz et al., 1968; Shepherd et al., 1976). A molecular weight of 192 000 was assumed to determine the molar concentrations of E_1 at pH 7.0; an extinction coefficient for E_1 of 0.23 μM^{-1} cm^{-1} at 280 nm was found by determining the protein concentration with the Lowry procedure using bovine serum albumin as the standard (Lowry et al., 1951).

The maleimide derivatives of E_1 were prepared as follows. The enzyme (15–35 μM) was mixed with a 10–40-fold excess of the reactant and allowed to react for 4 h at 4 °C with continuous stirring. The solution then was eluted through a Sephadex G-25 column (1 cm i.d. × 25 cm) equilibrated with the standard phosphate buffer. The fractions that contained protein (as determined by absorbance measurements at 280 nm) were pooled, the protein was precipitated with ammonium sulfate (70% saturation), and the solution was centrifuged at 19 000*g* for 15 min at 4 °C. The precipitate was suspended in the standard phosphate buffer and dialyzed extensively against the same buffer. It was observed that when an aged enzyme solution (1–2 months old, but still fully active) was used, the above procedure needed to be repeated for stoichiometric labeling.

The stoichiometry of E_1–NBM was found to be 1.75–1.95 mol of NBM/mol of E_1 dimer; the content of bound NBM was calculated assuming an extinction coefficient, ϵ, of 3.17×10^4 M^{-1} cm^{-1} at 310 nm as found for NBM (Kanoaka et al., 1968); the amount of protein was determined by measuring the absorbance at 280 nm and correcting for the NBM absorbance. The NBM extinction coefficient at 280 nm is 1.42×10^4 M^{-1} cm^{-1}. The protein absorbance is insignificant at 300 nm and above, although corrections for scattered light were made. The stoichiometry of E_1–NSM was 1.8–2.0 mol of NSM/mol of E_1 dimer using an extinction coefficient for NSM of 2.28×10^4 M^{-1} cm^{-1} at 350 nm, as found for the model compound NSM-Cys in ethanol and correcting for NSM absorbance at 280 nm ($\epsilon = 1.02 \times 10^4$ M^{-1} cm^{-1}) when determining the protein concentration. The stoichiometry of E_1–DDPM was about 2.0 mol of DDPM/mol of E_1 dimer using an extinction coefficient of 3.0×10^3 M^{-1} cm^{-1} at 440 nm as found for the model compound DDPM-*N*-acetylcysteine (Gold and Segal, 1964), and correcting for DDPM absorbance at 280 nm ($\epsilon = 1.53 \times 10^4$ M^{-1} cm^{-1}) in determining the protein concentration. Since the extinction coefficient of DDPM at 440 nm is small, the stoichiometry of E_1–DDPM was also checked by reacting the DDPM-modified enzyme (5.5 μM) with a 20-fold excess of NSM for 6 h. The amount of NSM incorporated was $\leq$0.2 mol of NSM/mol of E_1 dimer as assessed by comparison with the fluorescence of a mixture of unmodified E_1 (5.3 μM) and NSM reacted for 6 h under identical conditions. In addition to confirming the stoichiometry of the E_1–DDPM, this experiment also indicates that NSM and DDPM are reacting with the same sulfhydryl group(s).

Spectrophotometric Measurements. Absorbance measurements were done either with a Cary 118 or a Zeiss PMQII spectrophotometer. For recording difference spectra, rectangular quartz tandem cells (Pyrocell Manufacturing Co.) having a path length of 0.44 cm in each chamber were used with a constant instrumental slit width.

The steady-state fluorescence measurements were made with a Hitachi-Perkin-Elmer MPF-3 fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. The microcells (0.3 × 0.3 cm) were thermostated at the desired temperature, and, for low temperature work, dry nitrogen was circulated through the sample chamber to prevent condensation. Corrected emission spectra were either recorded utilizing the corrected spectrum accessories or computed by comparison with the spectrum of quinine bisulfate in 0.1 N H_2SO_4 with excitation at the appropriate wavelength at 25 °C (Melhuish, 1962; Chen, 1967). A comparative method (Parker and Rees, 1966) was used to determine the quantum yields of NBM, NSM, ANS, and thiochrome diphosphate bound to E_1. Equation 2 gives the ratio of quantum yields, Q_i, as a function of the area of the corrected emission spectrum, F_i, and the

TABLE I: Fluorescent Properties of Enzyme Derivatives.[a]

Enzyme Species	Q_D	τ_D (ns)	P_D	P_0
E_1-NBM	0.14	1.31	0.309	0.351[b]
E_1-NSM	0.14		0.392	0.419
E_1-ANS	0.87	20.3	0.357	0.396[c]
E_1-TCDP	0.25	2.58[d]	0.30[d]	0.34[d]
E_1-NBM-ANS	0.14	1.31	0.219	0.351[b]
E_1-NBM-TCDP	0.14			
E_1-ANS-DDPM	0.87	18.8	0.357	0.396[c]
E_1-TCDP-DDPM	0.26			

[a] 0.05 M potassium phosphate, pH 7.0, 5 °C; TCDP is thiochrome diphosphate. When two ligands are present, the fluorescent properties are for the first ligand written. [b] Cantley and Hammes (1976). [c] Stryer (1965). [d] Moe et al. (1974).

absorbance at the exciting wavelength, A_i, for two different fluorophores.

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1)/(1 - r_b/4) \quad (2)$$

The factor $(1 - r_b/4)$ is a correction for polarized emission (Shinitzky, 1972); r_b is the anisotropy of the fluorophore. Quinine bisulfate in 0.1 N H_2SO_4 was used as the standard and was assumed to have an absolute quantum yield of 0.70 (Scott et al., 1970) with an exciting wavelength of 350 nm at 25 °C. In the case of ANS, NSM, and thiochrome diphosphate, with exciting wavelengths of 378, 370 and 367 nm, respectively, appropriate corrections were made for the excitation wavelength dependence of the quantum yield of quinine (Chen, 1967). The absorbances of the samples at the exciting wavelengths were kept as low as possible ($\ll$0.05), and the areas of the corrected emission spectra were determined with a computer.

Since thiochrome diphosphate fluoresces in solution as well as when bound on the enzyme, the quantum yield of the bound fluorophore was computed, from the measured overall quantum yield, using the relationship

$$Q = X_f Q_f + X_b Q_b \quad (3)$$

where X_f and X_b are the mole fractions of free and enzyme-bound thiochrome diphosphate, and the Q's are the corresponding quantum yields. The quantum yield of the free thiochrome diphosphate was measured in a solution without E_1; in the presence of E_1, the concentration of the bound thiochrome diphosphate was calculated assuming a dissociation constant of 15 μM and assuming the absorbance spectrum of thiochrome diphosphate is unchanged when it binds to the enzyme (Moe and Hammes, 1974).

The absorbance of bound ANS was calculated using an extinction coefficient of 5.52×10^3 M^{-1} cm^{-1} at 378 nm. This extinction coefficient was calculated from the difference spectra of free ANS and mixtures of ANS and E_1. The dissociation constant characterizing the ANS-E_1 interaction, which is necessary for the calculation, was determined to be 3.19 μM by fluorescence titrations of E_1 with ANS as described under Results.

The fluorescence polarization measurements carried out with various fluorescent species were corrected for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating (Azumi and McGlynn, 1962) and for light scattering due to the protein.

The effect of various ligands upon the fluorescence of NSM or NBM bound to E_1 was recorded by titrating an enzyme solution (E_1-NSM or E_1-NBM) with the desired ligand, while monitoring the fluroescence of the probe. In the control experiments, NSM-Cys or NBM-Cys in the same buffer was titrated under identical conditions in order to correct for inner-filter and dilution effects.

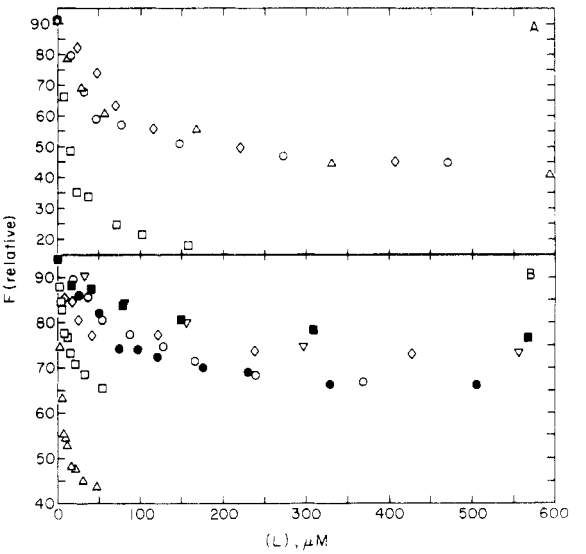

FIGURE 1: A plot of the relative fluorescence, F, vs. the total concentration of added ligand (L). The data were obtained by fluorescence measurements in 0.05 M potassium phosphate (pH 7.0) at 5 °C. (A) NSM bound on E_1 with 370-nm excitation, 435-nm emission; the ligands are (□) acetyl-CoA, (△) pyruvate, (○) thiamine diphosphate-$MgCl_2$, and (◇) phosphoenolpyruvate. The enzyme concentration was 1.25 μM, except with acetyl-CoA where the concentration was 0.75 μM. (B) NBM bound on E_1 with 310-nm excitation, 365-nm emission; the ligands are (△) ANS, (■) acetyl-CoA, (▽) GTP, (□) thiochrome diphosphate, (○) thiamine diphosphate-$MgCl_2$, (◇) pyruvate, (●) phosphoenolpyruvate. The enzyme concentration was 0.84 μM, except with phosphoenolpyruvate where the concentration was 1.46 μM.

Fluorescence Lifetime Measurements. Fluorescence lifetimes were measured with an ORTEC Model 9200 nanosecond fluorescence spectrophotometer (Matsumoto and Hammes, 1975). The excitation and emission wavelengths were selected with interference filters (Ditric Optics, Inc.) which exhibit maximum transmittance at the desired wavelengths (±5 nm). The samples were thermostated at 5 °C, and dry nitrogen was circulated through the sample chamber. All the decay spectra were accumulated for the same analysis time, and enzyme scattering spectra in the absence of fluorophores were subtracted as blanks. The data treatment and fitting procedures are described elsewhere (Shepherd and Hammes, 1976). Fluorescence depolarization measurements (Yguerabide, 1972) were carried out for ANS bound on E_1 at 5 °C, and the data were analyzed as previously described (Wu et al., 1975).

Results

Properties of E_1-Maleimide Derivatives. The specific activity of E_1, as measured with the ferricyanide assay in 0.02 M Tricine (pH 7.5), 30 °C, was 19–22 μmol of pyruvate oxidized/(mg h). The activity of E_1 was the same in the intact complex and was unchanged by modification with NBM, NSM, and DDPM.

The quantum yields and polarizations of NBM and NSM bound to E_1 are given in Table I along with the fluorescence lifetime of the NBM derivative. The excitation wavelengths were 310 and 370 nm for NBM and NSM, respectively, and the corresponding emission maxima were 365 and 435 nm. The limiting polarizations, P_0, at infinite viscosity are included in Table I.

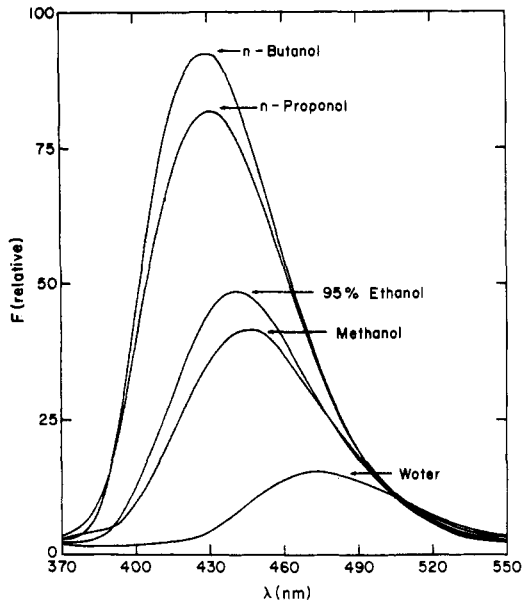

FIGURE 2: A plot of the uncorrected relative fluorescence of NSM-Cys in various solvents, F, vs. wavelength, λ. The data were obtained at 25 °C with 3.63 μM NSM-Cys and 360-nm excitation in 1-butanol, 1-propanol, 95% ethanol, methanol, and H_2O.

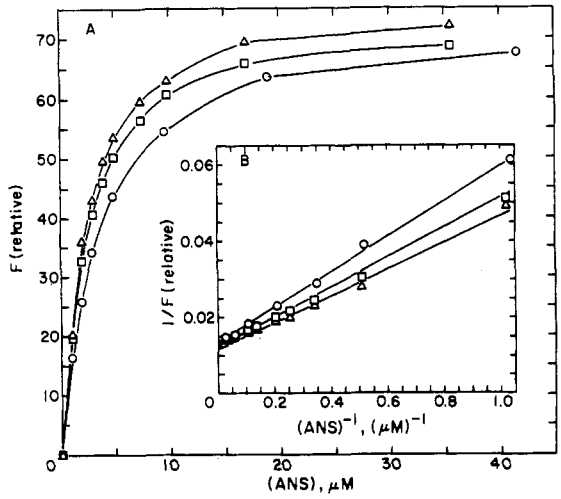

FIGURE 3: (A) A plot of the relative ANS fluorescence (378-nm excitation, 465-nm emission), F, vs. the total ANS concentration (ANS). The data were obtained by fluorescence titrations at 5 °C with 0.14–0.15 μM E_1 or modified E_1 in 0.05 M potassium phosphate (pH 7.0). (△) E_1, (□) E_1-NBM, and (○) E_1-DDPM. (B) A plot of $1/F$ vs. (ANS)$^{-1}$. The data were derived from Figure 3A. The lines are least-square fittings according to eq. 4.

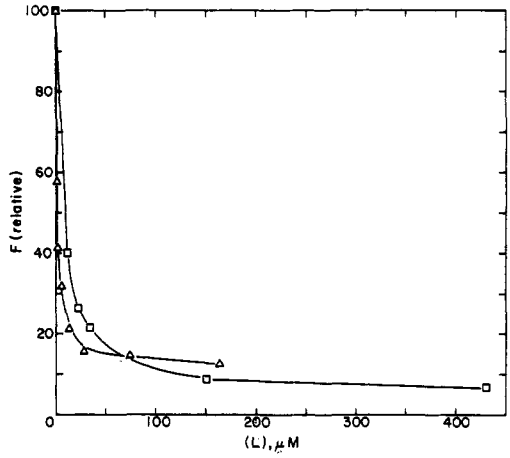

FIGURE 4: A plot of the relative ANS fluorescence (378-nm excitation, 465-nm emission), F, vs. the total concentration of added ligand (L). The data were obtained by fluorescence titrations at 5 °C in 0.05 M potassium phosphate (pH 7.0): (□) varying acetyl-CoA, 0.776 μM E_1 and 4.53 μM ANS; (△) varying quercetin, 0.95 μM E_1 and 5.06 μM ANS. The quercetin solution was in 95% ethanol and contained 1 mM dithiothreitol. The total amount of ethanol added for the entire titration was ~5% of the final volume.

The fluorescence of both NSM and NBM on E_1 was quenched when titrated with a variety of ligands known to bind to E_1, as shown in Figure 1A, B. The ligands used include the substrate pyruvate, the coenzyme thiamine diphosphate, the coenzyme analogue thiochrome diphosphate, the regulatory ligands acetyl-CoA, GTP, and phosphoenolpyruvate, and the acetyl-CoA analogue, ANS. This indicates that the environment of the fluorophores is altered by the binding of these ligands, since energy transfer is only possible for ANS and thiochrome diphosphate. To better understand the origin of the observed fluorescence changes, fluorescence emission spectra for NSM-Cys and NBM-Cys were determined in a variety of solvents. The uncorrected emission spectra of NSM-Cys in various solvents are shown in Figure 2. A decrease in the quantum yield is observed together with a shift of the emission maximum to the red as the solvent environment becomes more polar. In the case of NBM-Cys, a decrease in the quantum yield also was observed as the solvent became increasingly polar (1-butanol to H_2O) but the emission maximum was not shifted.

ANS Binding to E_1 and E_1-Maleimide Derivatives. The binding of ANS to E_1 and to E_1 modified by reaction with the maleimide derivatives was studied by following the change in fluorescence at 465 nm (378-nm excitation) when E_1 or its derivatives (0.14–0.15 μM) were titrated with ANS. The results are summarized in Figure 3A as plots of the relative ANS fluorescence vs. the total ANS concentration. If a single class of binding sites is assumed with an intrinsic dissociation constant K_D and the change in fluorescence is proportional to the amount of ANS bound,

$$1/F = 1/[f(E_0)] + K_D/[f(E_0)(\mathrm{ANS})] \qquad (4)$$

where F is the observed fluorescence, f is the proportionality constant which relates the concentration of bound ANS to the fluorescence, and (E_0) is the total enzyme concentration. In these experiments, the concentration of ANS is much larger than that of the enzyme so that the free and total ANS concentrations are essentially equal. Plots of the data according to eq 4 are shown in Figure 3B. A weighted least-squares analysis of the data gives a K_D of 3.02 μM for E_1, 3.17 μM for E_1-NBM, and 3.37 μM for E_1-DDPM. The average value of 3.19 μM was used for calculating the concentrations of free and bound ANS in all cases. The intercept $1/[f(E_0)]$ seems to be essentially the same in all cases, suggesting that the number of ANS binding sites and the fluorescent properties of ANS are not appreciably altered in the maleimide derivatives of E_1. It has been shown previously that the ANS binding to E_1 and to E_1 in the enzyme complex is the same and that two ANS binding sites are present per dimer of E_1 (Shepherd and Hammes, 1976).

As shown in Figure 4, the addition of acetyl-CoA or quercetin (a competitive inhibitor of ANS binding to chloroplast coupling factor; Cantley and Hammes, 1976) to an E_1 (0.776–0.95 μM)-ANS (4.53–5.06 μM) mixture results in

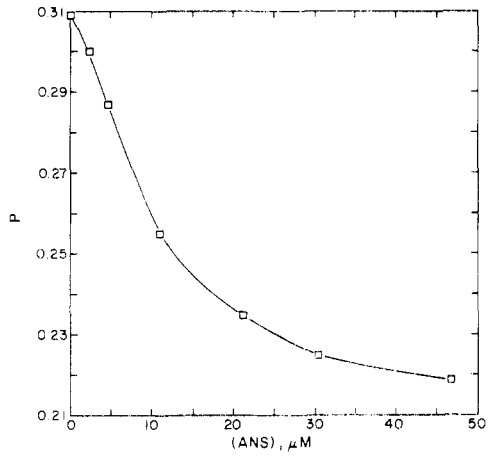

FIGURE 5: A plot of the polarization, P, of NBM bound on E_1 vs. the total ANS added (ANS). The data were obtained by fluorescence titration in 0.05 M potassium phosphate (pH 7.0) at 5 °C, 310-nm excitation, 365-nm emission, 1.04 μM E_1.

quenching of the ANS fluorescence. The concentration at which ANS fluorescence is 50% quenched is 9 μM for acetyl-CoA and 1.5 μM for quercetin. The shape of the emission spectrum of ANS did not change during these titrations. The difference spectrum between ANS and E_1-ANS mixtures also is essentially abolished at high acetyl-CoA concentrations. These results indicate that acetyl-CoA displaces ANS as found previously by binding measurements of ANS to E_1 in the enzyme complex (Shepherd and Hammes, 1976).

The quantum yield of ANS bound to E_1 (1.36 μM E_1, 13.0 μM ANS, 378-nm excitation) is given in Table I along with the fluorescence lifetime (4.35 μM E_1, 21.04 μM ANS, 378-nm excitation, 465-nm emission). The quantum yield of ANS bound to E_1-NBM (0.77 μM E_1-NBM, 21.1 μM ANS, 378 nm excitation) is 0.81. The steady-state fluorescence polarization of bound ANS (4.35 μM E_1, 21.0 μM ANS, 378-nm excitation, 465-nm emission) is given in Table I along with the limiting polarization. The steady-state fluorescence polarization of ANS bound to E_1-NBM is the same. However, as shown in Figure 5, the polarization of bound NBM decreases markedly when the enzyme is titrated with ANS; at saturating ANS concentrations, a limiting value of 0.219 is reached. Anisotropy depolarization measurements with ANS bound to E_1 (28.4 μM E_1, 338 μM ANS) gave a rotational correlation time of 100 ns.

Binding of Thiochrome Diphosphate to E_1 and E_1-Maleimide Derivatives. In order to calculate the concentration of thiochrome diphosphate bound on E_1, a dissociation constant of 15 μM was used, as reported for the binding of thiochrome diphosphate to the pyruvate dehydrogenase complex. The validity of this value was checked by using it to calculate the mole fractions of bound thiochrome diphosphate in several E_1-thiochrome diphosphate mixtures; the fluorescence polarization of these mixtures agreed to within 10% with the previously published polarizations of thiochrome diphosphate bound to the complex (Moe et al., 1974). A similar result was obtained with thiochrome diphosphate bound to E_1-DDPM and to E_1-NBM. This indicates that the binding of thiochrome diphosphate to E_1 is not altered when the enzyme is separated from the complex or when it is modified with DDPM or NBM.

The quantum yields (367-nm excitation) and polarizations (367-nm excitation, 435-nm emission) of bound thiochrome diphosphate on E_1 and on E_1-DDPM are included in Table I. The quantum yield of thiochrome diphosphate in the absence of protein is 0.23. The corrected emission maximum is shifted from 445 to 440 nm in the presence of E_1 and E_1-DDPM (367-nm excitation).

Energy-Transfer Measurements. The efficiency, E, of energy transfer from a fluorescent donor molecule to an absorbing acceptor molecule is given by

$$E = 1 - Q_{D\rightarrow A}/Q_D = 1 - \tau_{D\rightarrow A}/\tau_D \qquad (5)$$

where Q and τ refer to the quantum yield and fluorescence lifetime of the donor molecule in the presence (D→A) or absence of acceptor (D). Although the fluorescence of NBM bound on E_1 is quenched by a variety of ligands, NBM can be used as a donor for fluorescence energy transfer if the acceptor molecules are appropriately selected. Both ANS and thiochrome diphosphate bind to E_1 at different, but specific, sites, and the binding is unaltered by NBM or DDPM modification of E_1. Furthermore, the absorbtion spectra of ANS and thiochrome diphosphate overlap well with the corrected emission spectrum of NBM and their corrected emission spectra overlap well with the absorption spectrum of DDPM.

To determine the energy transfer between NBM and ANS or between NBM and thiochrome diphosphate, E_1-NBM was titrated with the acceptor (ANS or thiochrome diphosphate). The fluorescence of both the donor (365-nm emission) and the acceptor molecule (465-nm emission for ANS, 435-nm emission for thiochrome diphosphate) were recorded with an excitation wavelength of 310 nm, which corresponds to the wavelength of maximum NBM excitation. The acceptor fluorescence was corrected for donor fluorescence as well as for direct acceptor excitation at 310 nm. Although the NBM fluorescence is quenched, the wavelength dependence of its emission spectrum was found to be unchanged by the presence of the acceptor. The implicit assumption has been made that the acceptor excitation spectrum is not altered by the donor. To ascertain if energy transfer occurs, an increase in the fluorescence of ANS or thiochrome diphosphate was looked for when NBM is excited; an enhancement of fluorescence was not observed. A generous estimate of the experimental uncertainty is 10%, so that the efficiency of energy transfer is ≤ 0.1.

To check the above results and also to check for possible orientational effects in the energy transfer process, a different pair of donor and acceptor molecules was used at the same sites: ANS and thiochrome diphosphate were used as donors while DDPM was utilized as the acceptor molecule. The fluorescence lifetime of ANS bound on E_1-DDPM is given in Table I, and comparison with the lifetime of bound ANS on the absence of DDPM gives an energy transfer efficiency of 0.074, which is probably significant although it is similar to the estimated uncertainty. The quantum yield of thiochrome diphosphate bound to E_1 was not altered by modification of E_1 with DDPM so that the efficiency of energy transfer from thiochrome diphosphate to DDPM is ≤ 0.1.

The measurement of energy transfer between the acetyl-CoA (or ANS) binding site and the thiochrome diphosphate (or thiamine diphosphate) binding site was not possible, since the binding of ANS results in dissociation of thiochrome diphosphate from E_1 as indicated by a decrease in the thiochrome diphosphate polarization.

The distance or minimum distance between the donor and acceptor molecules can be calculated from the energy transfer efficiencies summarized in Table II by use of eq 6:

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

where R is the distance between the donor and acceptor and

$$R_0 = 9.79 \times 10^3 (Q_D K^2 J n^{-4})^{1/6} \text{ Å} \quad (7)$$

In the equation for R_0, Q_D is the quantum yield of the donor (given in Table I), K^2 is the dipole–dipole orientation factor, J is the integral of the spectral overlap for the normalized donor fluorescence and acceptor absorbance spectra, and n is the refractive index of the medium (1.4). The values of R_0 given in Table II were calculated assuming $K^2 = \frac{2}{3}$, which is the value for freely rotating donor and acceptor molecules; the overlap integral was calculated as previously described (Cantley and Hammes, 1975). The absorbance spectrum of DDPM–*N*-acetylcysteine in 0.1 N sodium phosphate, pH 7.0 (Gold and Segal, 1964), was used in calculating the overlap integral. The values of R calculated for the four donor–acceptor pairs are included in Table II.

TABLE II: Summary of Energy Transfer Experiments.[a]

Enzyme Species	R_0 (Å)	E	R (Å)
E_1-NBM-ANS	24.4	≤0.1	≥35
E_1-NBM-TCDP	29.0	≤0.1	≥42
E_1-ANS-DDPM	32.2	0.074	49
E_1-TCDP-DDPM	27.7	≤0.1	≥39

[a] 0.05 M potassium phosphate, pH 7.0, 5 °C; TCDP is thiochrome diphosphate.

Discussion

The modification of a single sulfhydryl group on the polypeptide chain of E_1 with NBM, NSM, or DDPM apparently does not alter the enzyme conformation appreciably, since the enzymatic activity remains the same and the binding constants for ANS on the acetyl-CoA site and for thiochrome diphosphate on the thiamine diphosphate site are unchanged. Similarly, the enzyme activity and the binding constant for ANS and thiochrome diphosphate on E_1 in the intact multienzyme complex and to the isolated enzyme are the same. However, the fluorescence of NBM or NSM when bound to the sulfhydryl group is a very good indicator of the binding of substrates, cofactors, inhibitors and activators to the enzyme. The fluorescence emission spectra suggest that NBM and NSM are in hydrophobic environments and that ligand binding exposes them to a more polar environment. This interpretation is also supported by the marked decrease in NBM fluorescence polarization accompanying the binding of ANS. In spite of the sensitivity of the fluorescence to ligand binding, the energy transfer measurements summarized in Table II indicate the catalytic site and the acetyl-CoA site are quite far from the fluorescence probes (≥40 Å and ~50 Å, respectively). Thus, the binding process for all of the ligands must involve conformational changes that extend over large distances from the binding loci.

The energy transfer measurements are difficult because of the quenching of NBM fluorescence associated with ligand binding. However, since the amount of quenching is the same with both a potential energy acceptor and a nonacceptor (thiochrome diphosphate vs. thiamine diphosphate) and the ANS fluorescence is not enhanced when it is used as an energy acceptor, it is unlikely the quenching can be attributed to energy transfer. This interpretation is also consistent with the lack of fluorescence enhancement of thiochrome diphosphate due to energy transfer from NBM, even though it is difficult to detect small changes in the fluorescence of the bound thiochrome diphosphate because of the strong fluorescence background contribution of the free fluorophore.

The usual uncertainty in K^2 exists when calculating the distance between donors and acceptors. Since the observed polarizations (P) are all less than the limiting polarizations (P_0) (Table I), some rotational motion of the fluorescent probes must exist. Estimates of the mean rotational correlation times, ϕ, which are an indication of the relative mobility of the fluorescent probes, may be obtained by using eq 8 (Weber, 1952)

$$\phi = \tau / [(1/P - 1/3)/(1/P_0 - 1/3) - 1] \quad (8)$$

In this equation, τ is the fluorescence lifetime, P is the measured polarization, and P_0 is the polarization at infinite viscosity. Using the values of P, P_0, and τ in Table I, ϕ = 8.51 ns for NBM, ϕ = 148.5 ns for ANS, and ϕ = 17.2 ns for thiochrome diphosphate. For NBM and thiochrome diphosphate, the computed mean rotational correlation times are much shorter than the corresponding time measured for E_1 (100 ns), indicating considerable freedom of the fluorophores to rotate on the enzyme. Although the motion of the ANS appears to be restricted, the corresponding donor (NBM) or acceptor DDPM (bound at the same site as NBM) appears to be quite mobile. Furthermore, the calculated distances obtained with two different sets of probes are self-consistent. Thus, the assumption that $K^2 = \frac{2}{3}$ is unlikely to be seriously in error.

All of the calculations assume a single donor–acceptor pair; however, since E_1 is a dimer (Eley et al., 1972), two acceptors and two donors are actually present. If the interaction between energy donors and acceptors on different polypeptide chains is important, the actual distances would be somewhat longer than calculated; therefore, the distances in Table II can be regarded as lower bounds.

The rotational correlation time of 100 ns measured for ANS bound on E_1 agrees quite well with the calculated value of 117 ns. This calculation assumed a molecular weight of 192 000 (Eley et al., 1972), a specific volume of 0.731 mL/g, hydration of 0.2 g of water/g of protein, and utilized the equation appropriate for a hydrated spherical molecule (Yguerabide et al., 1970).

The results presented here provide evidence for a conformational change accompanying the binding of a variety of ligands to E_1 with the covalently bound fluorescence probe being quite distant from the binding sites. The fluorescent probes are now being utilized for quantitative thermodynamic and kinetic measurements of ligand binding to E_1.

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Lipoprotein Lipase. Isolation and Characterization of a Second Enzyme Species from Postheparin Plasma†

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ABSTRACT: A lipoprotein lipase species (mol wt 69 250) has been isolated from rat postheparin plasma, which differs from the low-molecular-weight species previously characterized in its amino acid composition and hexosamine content, and in its lower affinity for triglyceride-rich lipoprotein substrates. However, both enzymes are activated by the same coprotein (C-terminal glutamic acid, apo-C-2) from human very low density lipoprotein and have a similar specificity for lipid esters. Neither purified enzyme is activated by heparin. Both are inhibited by molar sodium chloride. Both enzyme species can be recovered from the same plasma samples. The possible relationship of these proteins to the different functional lipoprotein lipase activities of muscle and adipose tissues is discussed.

Lipoprotein lipase at the vascular surface of different tissues is functionally heterogeneous. In experiments with perfused heart (Fielding and Higgins, 1974) and adipose tissues (Fielding, 1976), kinetic studies of the membrane-supported lipase suggested the presence of enzyme sites with high and low affinities, respectively, for reaction with natural lipoprotein substrates. The lipase with high substrate affinity, released by heparin from the perfused heart, has been isolated and characterized from postheparin plasma (Fielding et al., 1974). The purpose of the present study was to identify and isolate from postheparin plasma a component corresponding to the low affinity lipoprotein lipase species.

Experimental Section

Lipoprotein Preparation. Very low density lipoproteins were isolated from the plasma of male Sprague-Dawley rats (300–350 g) by ultracentrifugal flotation (Havel et al., 1955). Plasma containing 0.1% Na_2EDTA[1] (pH 7.4) was centrifuged at $1.6 \times 10^5 g$-min and the floating lipoprotein material (S_f, flotation index, > 400) (Dole and Hamlyn, 1962) was discarded. The infranatant fraction was centrifuged for $4.86 \times 10^6 g$-min and the floating triglyceride-rich lipoprotein, after recentrifugation under the same conditions, was used as lipase substrate in the experiments described below.

Chylomicrons were prepared from rat intestinal lymph obtained from animal donors bearing a cannula in the mesenteric lymph duct and in the duodenal lumen (Fielding and Higgins, 1974). Synthetic triglyceride–lecithin dispersion (Intralipid, Cutter Laboratories, Oakland, Calif.) (2.5% w/v triglyceride) was infused at a flow rate of 2 mL/h into the duodenum. Lymph collected for up to 18 h in ice-cooled tubes was centrifuged at $9.5 \times 10^4 g$-min to remove the largest particles, then the infranatant fraction was recentrifuged at 1.6×10^5 g-min to float the major chylomicron fraction. This was recentrifuged under the same conditions. Triglyceride-depleted plasma was prepared by removal of very low density lipoprotein ($d < 1.006$ g cm^{-3}) from whole plasma. The infranatant solution, containing residual plasma proteins concentrated two- to threefold (Fielding and Higgins, 1974) was dialyzed against 500 volumes of Krebs–EDTA buffer (pH 7.4).

Preparation of Lipoprotein Polypeptides. Human very low density lipoprotein obtained by centrifugation from the plasma of normal donors was delipidated with mixtures of diethyl ether and ethanol (Shore and Shore, 1969). Low-molecular-weight polypeptides were separated from total apoprotein by gel filtration on Sephadex G-150 (Pharmacia, Piscataway, N.J.) in

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[1] Abbreviations used: Na_2EDTA, ethylenediaminetetraacetic acid, sodium salt; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.