Koike, M., Reed, L. J., and Carroll, W. R. (1963), *J. Biol. Chem. 238*, 30.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Moe, Jr., O. A., Lerner, D. A., and Hammes, G. G. (1974), *Biochemistry 13*, 2552.
Reed, J. J., and Mukherjee, B. B. (1969), *Methods Enzymol. 13*, 55.
Risinger, G. E., and Parker, P. N. (1965), *Experientia 21*, 305.
Schwartz, E. R., Old, L. O., and Reed, L. J. (1968), *Biochem. Biophys. Res. Commun. 31*, 495.
Viscontini, M., Bonnetti, G., and Karrer, P. (1949), *Helv. Chim. Acta 32*, 1478.
Vogel, O., Hoehn, B., and Henning, U. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1615.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wittorf, J. H., and Gubler, C. J. (1970), *Eur. J. Biochem. 14*, 53.

Fluorescence Energy Transfer between the Thiamine Diphosphate and Flavine Adenine Dinucleotide Binding Sites on the Pyruvate Dehydrogenase Multienzyme Complex†

Owen A. Moe, Jr.,‡ D. A. Lerner, and Gordon G. Hammes*

ABSTRACT: Thiochrome diphosphate and flavine adenine dinucleotide, which bind to the active sites of the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase enzymes of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*, have been utilized as a donor–acceptor pair for fluorescence energy transfer. Results from steady-state and nanosecond lifetime fluorescence measurements are consistent with a decrease in both the quantum yield and the fluorescence lifetime of enzyme-bound thiochrome diphosphate when flavine adenine dinucleotide is present at its binding site. The results obtained have been analyzed in terms of an energy-transfer mechanism, and the measured efficiency of transfer was found to be 7.8%. This corresponds to an apparent distance between sites of approximately 45 Å. Uncertainty concerning the relative geometrical orientation of the donor and acceptor transition dipoles gives rise to some uncertainty in this distance, but consideration of the dependence of the distance on the orientation of the dipoles indicates the possible range of distances is about 30–60 Å. The fluorescence and absorption properties of flavine adenine dinucleotide are not altered by the binding of ligands at the active sites of other enzymes in the multienzyme complex, indicating very little interaction between the active sites of different enzymes in the complex. These results place some limitations on the catalytic mechanism for this multienzyme complex.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* has been shown to exist in a regularly packed arrangement of three enzymes (Koike *et al.*, 1963; Willms *et al.*, 1967). The enzymes are pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. These enzymes have been postulated to catalyze the sequence of consecutive reactions in the oxidative decarboxylation of

$$\text{pyruvate} + E_1[\text{TDP}] \rightleftharpoons CO_2 + E_1[\text{hydroxyethyl-TDP}] \quad (1)$$

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

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

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

pyruvate given in eq 1–4 (Koike *et al.*, 1960). In this scheme, E_1, E_2, and E_3 refer to the pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase enzymes, respectively; TDP, Lip-$(S)_2$, and Lip-$(SH)_2$ refer to thiamine diphosphate and the oxidized and reduced forms of lipoic acid, respectively. The brackets indicate tightly bound enzyme intermediates.

This proposed catalytic sequence requires the interaction of different forms of the lipoic acid moiety with active sites on all three component enzymes. This implies that a critical topographical relationship exists between the different active sites which is necessary for optimal catalytic activity. Lipoic acid is covalently attached to a lysine ϵ-amino group (Nawa *et al.*, 1960) and forms thereby an effective "arm" of about 14 Å from the protein backbone to the reactive dithiolane ring of the lipoic acid (Koike *et al.*, 1963). Rotation of the lipoic acid moiety from site to site is a possible mechanism for the postulated catalytic sequence (Koike *et al.*, 1963). This, of course, places the restraint on the system that all participating active sites must be within a circle approximately 28 Å in diameter.

The work presented in this paper is an attempt to test the above hypothesis by measurement of the distance between two of the active sites involved in the catalytic sequence using fluorescence singlet–singlet energy-transfer measurements (Steinberg, 1971; Stryer and Haugland, 1967). The efficiency of energy transfer, E, between a donor and acceptor molecule is given by

$$E = 1 - Q_T/Q_0 = 1 - \tau_T/\tau_0 \quad (5)$$

† From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received December 3, 1973.* This work was supported by a grant from the National Institutes of Health (GM 13292).

‡ National Institutes of Health Postdoctoral Fellow, 1971–1973 (GM 50359).

In this equation Q and τ refer to the quantum yield and excited-state lifetime of the donor molecule. The subscripts T and 0 refer to measurements made in the presence and absence of acceptor molecule, respectively.

The efficiency of energy transfer has been related to the distance between donor and acceptor molecules, r, by a theoretical treatment of Förster (1959) (eq 6). In eq 6 R_0 is

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

the distance of separation at which the efficiency of energy transfer is 50% and is given by

$$R_0 = (9.79 \times 10^3)(JK^2Qn^{-4})^{1/6} \quad (7)$$

In this equation, K^2 is a dipole–dipole orientation factor, Q is the quantum yield of the donor molecule, n is the refractive index of the medium, and J is the integral of spectral overlap which can be expressed as

$$J = \int F_\nu E_\nu d\nu/\nu^4 \quad (8)$$

where $E(\nu)$ is the molar extinction coefficient of the energy acceptor at wave number ν and $F(\nu)$ is the fluorescence intensity of the energy donor, normalized to its total integrated spectrum, using a wave number scale.

In the work presented here thiochrome diphosphate, a fluorescent analog of thiamine diphosphate which has been shown to bind tightly and specifically to the thiamine diphosphate site (Moe and Hammes, 1974), was used as a donor molecule for energy-transfer measurements. The tightly bound FAD on the dihydrolipoyl dehydrogenase component enzyme was used as the energy acceptor. The results suggest the two sites are about 45 Å apart, with a possible range of 30–60 Å. The range is due to the uncertainty in the relative orientations of the acceptor and donor molecules.

Experimental Section

Materials. Thiamine hydrochloride and FAD were purchased from Sigma Chemical Corp. Quinine bisulfate was obtained from Eastman Kodak. Pyruvate dehydrogenase complex was purified from *E. coli* (Reed and Mukherjee, 1969). The synthesis of thiochrome diphosphate and procedures for removal of thiamine diphosphate and FAD from the enzyme complex were previously described (Moe and Hammes, 1974). All other chemicals were the best available commercial products. Distilled, deionized water was used for the preparation of all solutions.

Steady-state fluorescence measurements were made with an Hitachi-Perkin-Elmer MPF-3 fluorescence spectrophotometer. Measurements of the fluorescence polarization, P, were carried out using polarizers for the excitation and emission light paths. Correction for unequal transmission of the horizontal and vertical components of polarized light was made according to eq 9 (Azumi and McGlynn, 1962). The terms

$$P = \frac{V_v - H_v[V_h/H_h]}{V_v + H_v[V_h/H_v]} \quad (9)$$

V and H refer to the intensities of the emitted light measured with the emission polarizers in the vertical and horizontal directions, respectively. The subscripts, v and h, refer to the orientation of the excitation polarizer.

Measurements of the limiting polarization, P_0, were made at 20° using sucrose to vary the viscosity of the medium. Plots of $1/P - 1/3$ *vs.* T/η (Perrin, 1926) were extrapolated to $T/\eta = 0$ to determine P_0, using the following relationship.

$$(1/P - 1/3) = (1/P_0 - 1/3)(1 + RT\tau/\eta V) \quad (10)$$

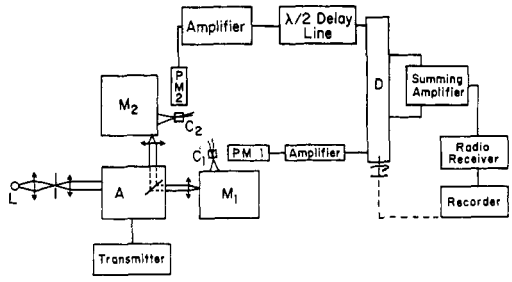

FIGURE 1: Block diagram of the phase fluorimeter used for the measurement of fluorescence lifetimes. In this diagram L is the lamp, A is the electrooptic modulator and beam splitter, M_1 and M_2 are monochromators, C_1 and C_2 are reference and sample cells, and D is a continuously variable delay line.

In this equation R is the gas constant, T is the absolute temperature, τ is the lifetime of the excited state, V is the molar volume, and η is the viscosity of the solvent.

The corrected emission spectrum of thiochrome diphosphate, which was necessary for determinations of quantum yield and the spectral overlap integral, was calculated from comparison of its uncorrected spectrum with that of quinine bisulfate, a fluorescence standard with a known emission spectrum (Berlman, 1965). The quantum yield of thiochrome diphosphate, φ_{TCDP}, is given by (Parker and Rees, 1964)

$$\varphi_{TCDP} = \varphi_{QBS}\frac{\text{Area}_{TCDP}}{\text{Area}_{QBS}}\frac{\text{ABS}_{QBS}}{\text{ABS}_{TCDP}} \quad (11)$$

The areas refer to the integrated, corrected emission spectra (on a wave number scale) of thiochrome diphosphate (TCDP) and quinine bisulfate (QBS) and ABS is the absorbance at the wavelength of excitation, which was 350 nm. The quantum yield of quinine bisulfate, φ_{QBS}, was taken to be 0.55 (Berlman, 1965).

Fluorescence Lifetime Measurements. The fluorescence lifetime measurements were carried out with a phase fluorimeter having a time resolution of about 20 psec. The instrument was a modification of that described by Müller *et al.* (1965). A new feature is that the amplitude modulated beam is split into two equivalent beams, either of which can be used as reference or working channel. This symmetry eliminates a number of possible sources of undesirable phase shifts and permits differential measurement of ultra-fast lifetimes. A complete description of this instrument will be presented elsewhere (D. A. Lerner and A. C. Albrecht, in preparation).

A block diagram of the measuring apparatus is shown in Figure 1. The light of a 2.6-kW Xe arc lamp is collimated through an electrooptic light modulator and is split into two beams. Each beam goes through a Bausch and Lomb 500-mm grating monochromator. The instrument is initially zeroed by putting a light-scattering agent (Ludox) in both beams. The measurement is made by replacing the scattering agent in one of the beams with the fluorescent sample. Both fluorescence and scattering are observed at right angles to the original beams. The light is collected on a small area of the cathode of a RCA 8575 photomultiplier. The signals are preamplified, balanced in intensity, phase shifted (one in a continuously variable delay line, the other by a half-wave delay line) and are added in a sensitive radio receiver, converted to a dc signal, and sent to a recorder. The phase delay was measured by varying the delay of the reference signal until the two signals are 180° out of phase. If the sinusoidally modulated light has a frequency f, the time dependence of the intensity, $I(t)$, is

$$I(t) = A + B\cos(2\pi ft) \quad (12)$$

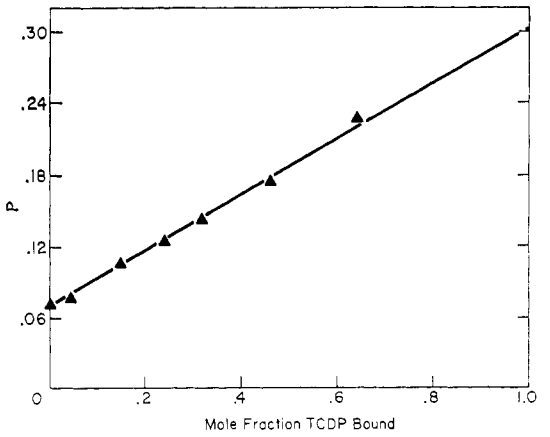

FIGURE 2: A plot of the fluorescence polarization of thiochrome diphosphate, P, *vs.* the mole fraction of thiochrome diphosphate bound to the pyruvate dehydrogenase complex. The solutions contained 0.01 M potassium phosphate (pH 6.2), 0.5 mM $MgCl_2$, and thiochrome diphosphate and pyruvate dehydrogenase complex in varying concentrations.

The fluorescence emission, $F(t)$, from molecules excited by this light is (Duschinsky, 1929)

$$F(t) = a + b \cos \phi \cos (2\pi ft - \phi) \qquad (13)$$

where $tg\phi = 2\pi f\tau$ and τ is the lifetime of the fluorescence. Thus the fluorescence lifetime is directly related to the measured phase shift.

The crystal controlled modulation frequencies used were 14.7925 and 22.1887 MHz. The determination of the phase shift is accurate to $\pm 0.05°$, which corresponds to a value of $\Delta\tau/\tau$ of 1% or less for τ varying between 0.85 and 33 nsec at a modulation frequency of 14.7925 MHz. Cutoff and band pass filters were used to keep the scattered light to 1% or less. Light was scattered off Ludox at the wavelength corresponding to the emission maximum (440 nm) with the cutoff filter in position in both channels. When the Ludox is replaced by the sample in one channel and the exciting wavelength is used, a time delay occurs that is directly related to τ. The exciting light, 367 nm, was linearly polarized at an angle of 35° with respect to the vertical and all of the emission was viewed to ensure that photoselection does not distort the observed τ (Spencer and Weber, 1970). The value of τ was identical with both modulating frequencies.

Results

Measurements of Energy Transfer. Measurements of energy transfer between the protein bound thiochrome diphosphate and FAD were made using both steady-state fluorescence measurements and nanosecond lifetime measurements. The experimental conditions for all measurements were 0.01 M potassium phosphate (pH 6.2), 0.2 mM $MgCl_2$, 20 μM thiochrome diphosphate, 0–8.4 mg/ml of pyruvate dehydrogenase complex, and a temperature of 10°. Due to the high absorbance of these solutions at the wavelength of excitation (367 nm), triangular cells, designed to significantly decrease the excitation optical path, were used. The mole fraction of bound thiochrome diphosphate was calculated from the known binding parameters (Moe and Hammes, 1974) and was also directly checked by fluorescence polarization measurements, since polarization was found to be a linear function of the mole fraction of bound thiochrome diphosphate (Figure 2).

Steady-state fluorescence measurements were made with thiochrome diphosphate bound to pyruvate dehydrogenase

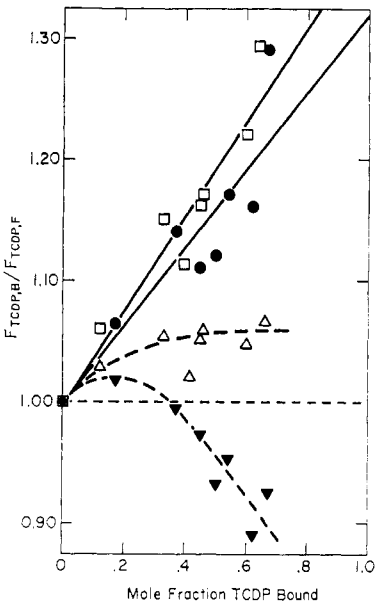

FIGURE 3: A plot of the ratio of thiochrome diphosphate fluorescence when partially bound to pyruvate dehydrogenase complex to its fluorescence free in solution $F_{TCDP,B}/F_{TCDP,F}$, *vs.* the mole fraction of thiochrome diphosphate bound to the protein. The solutions contained 0.01 M potassium phosphate (pH 6.2), 0.2 mM $MgCl_2$, 20 μM thiochrome diphosphate, and pyruvate dehydrogenase complex in varying concentrations. Enzyme complex containing 100% FAD: (▼) uncorrected for absorption of emitted light; (●) corrections applied. Enzyme complex containing 33% FAD; (△) uncorrected for absorption of emitted light; (□) corrections applied. The solid lines for ● and □ were calculated by linear regression analysis.

complex containing 100% FAD and 33% FAD. Excitation was at 367 ± 4 nm and emission was measured at 440 ± 10 nm. The absorbance of the solutions was measured at 440 nm and was used to correct for the attenuation of the emitted light due to self-absorbance. The effective emission path length for the triangular cell was determined to be 0.475 cm. No correction for the attenuation of the exciting radiation was made because triangular cells were used. The fluorescence intensity at 440 nm for each sample was normalized to the case where no enzyme was present (mole fraction bound equals zero).

A plot of the normalized fluorescence intensity *vs.* the mole fraction of thiochrome diphosphate bound is shown in Figure 3. The two lower curves represent the measurements uncorrected for emission attenuation, while the upper curves are their corrected counterparts. Because the attenuation correction is very large, quantitative deductions cannot be made from the steady-state measurements. Qualitatively, however, the following points should be noted. The fluorescence intensity of thiochrome diphosphate increases as the mole fraction of thiochrome diphosphate bound increases. Extrapolation to a mole fraction bound equal to 1.0 would give a fluorescence enhancement of the bound ligand relative to the free ligand of *ca.* 30–50%. Secondly, the enhancement obtained with the enzyme complex containing 33% FAD appears to be slightly greater than the corresponding points obtained with enzyme complex containing 100% FAD. In both cases, a slight shift in the maximum wavelength of emission (uncorrected spectra) from 442 to 438 nm was observed to accompany the thiochrome diphosphate binding.

Since excited-state lifetime measurements depend only on the time constant of fluorescence decay and not on the amplitude of the fluorescence signal, large corrections of the

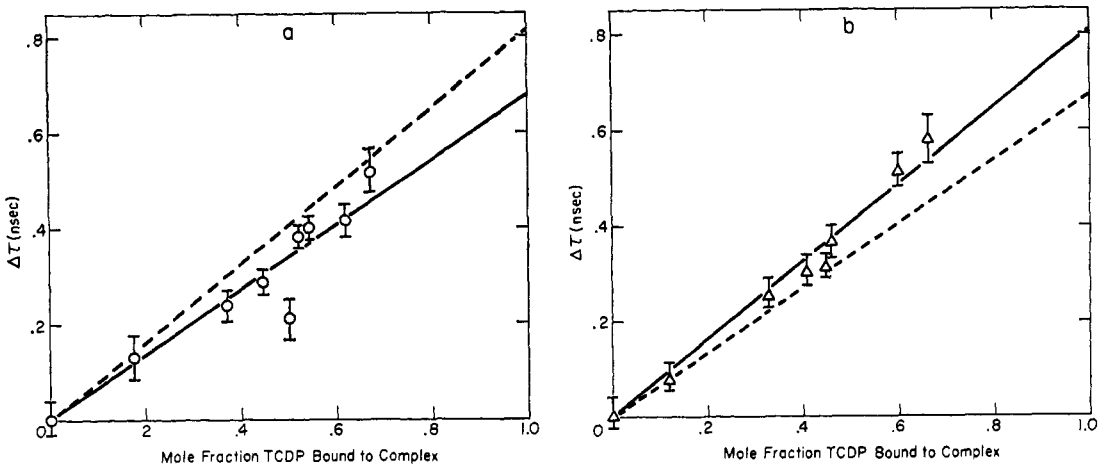

FIGURE 4: A plot of $\Delta\tau$, the difference in excited state lifetimes between enzyme bound and free thiochrome diphosphate, *vs.* the mole fraction of thiochrome diphosphate bound. The solutions contained 0.01 M potassium phosphate (pH 6.2), 0.2 mM $MgCl_2$, 20 μM thiochrome diphosphate, and pyruvate dehydrogenase complex in varying concentrations. The enzyme contained either 100% FAD (O, a) or 33% FAD (Δ, b). The error bars represent standard deviations for 4–12 individual measurements. The solid lines were calculated by linear regression analysis of the data. Dotted lines refer to the fit obtained for the data in the accompanying graph.

experimental data due to self-absorption by the sample are not necessary. For this reason, measurements of the fluorescence lifetime of thiochrome diphosphate were made.

Nanosecond lifetime measurements were made for thiochrome diphosphate bound to pyruvate dehydrogenase complex containing 100% FAD and 33% FAD. Measurements with a standard 20 μM thiochrome diphosphate solution, containing all components except pyruvate dehydrogenase complex (*i.e.*, mole fraction bound equal to zero) were made immediately before and after measurements with each sample containing protein. The results are expressed as the lifetime of the thiochrome–protein mixture minus that of the standard solution (designated as $\Delta\tau$). The absolute fluorescence lifetime of thiochrome diphosphate was determined to be 1.70 nsec.

Plots of $\Delta\tau$ *vs.* the mole fraction of thiochrome diphosphate bound for the enzyme complex with 100% FAD and 33% FAD are shown in Figure 4. Experimental measurements were made over a period of 5 weeks, and points on Figure 4a and b corresponding to approximately the same mole fraction bound were measured on the same day. The lifetimes measured for the enzyme with 33% FAD were consistently higher than those for the enzyme with 100% FAD. The extrapolated intercept at a mole fraction bound equal to 1.0 was obtained by a linear regression analysis of the data. The results obtained for $\Delta\tau$ were 0.81 $\pm$ 0.06 and 0.68 $\pm$ 0.10 nsec for the complex with 33% FAD and 100% FAD, respectively. This corresponds to a difference of 130 psec in the fluorescence lifetime of the thiochrome diphosphate bound to the two different forms of the enzyme. The experimental uncertainty in individual measurements is estimated to be $\pm$30 psec.

Fluorescence Polarization Measurements. The correlation of efficiency of energy transfer with the distance between donor and acceptor molecules requires knowledge of the orientation of the emission and absorption dipoles with respect to each other (*i.e.*, a knowledge of the K^2 term in eq 7). If either of the molecules involved in energy transfer is free to rotate at a rate much greater than the decay of the donor excited state, an average value of K^2 ($^2/_3$) can be used (Förster, 1959).

A qualitative indication of the rotational mobility of a fluorescent molecule can be obtained from fluorescence polarization measurements. Determinations of the limiting polarization values, P_0, were made for thiochrome diphosphate (367-nm transition) and FAD (450- and 360-nm transitions). These results are given in Table I. P_0 depends only on the angle, θ, between the absorption and emission dipoles (Weber, 1966) (eq 14), and is reached experimentally when the rate of rotational motion of a molecule is negligibly small in comparison to the rate of excited-state decay.

$$P_0 = \frac{3\cos^2\theta - 1}{3 + \cos^2\theta} \qquad (14)$$

Table I also includes values of polarization for thiochrome diphosphate and FAD in water as well as when bound to the enzyme. The polarization value for thiochrome diphosphate bound to the enzyme was determined by measuring the polarization of thiochrome diphosphate as a function of mole fraction bound and extrapolating to a mole fraction of 1.0 (see Figure 2). The mole fraction bound was calculated using previously determined values for the thiochrome diphosphate dissociation constant and the number of binding sites (Moe and Hammes, 1974). FAD is tightly incorporated in the enzyme complex so that an extrapolation was not needed.

Comparison of the limiting polarization in sucrose with the polarization of the protein-bound molecules indicates that FAD is very rigidly bound, while thiochrome diphosphate may have a small amount of rotational freedom.

Discussion

Although the large inner filter corrections which were necessary in the steady-state fluorescence intensity measure-

TABLE I: Fluorescence Polarization Measurements of Free and Enzyme Bound Ligands.

Ligand	Excitation Wavelength (nm)	P_{H_2O}	P_0[a]	P_{enzyme}
Thiochrome diphosphate	367	0.06	0.34	0.30[b]
FAD	450	0.04	0.44	0.46
FAD	360	0.02	0.25	0.26

[a] P_0 is polarization extrapolated to infinite viscosity. [b] Obtained by measuring P *vs.* mole fraction of ligand bound and extrapolating to 100% bound (see Figure 2 and text).

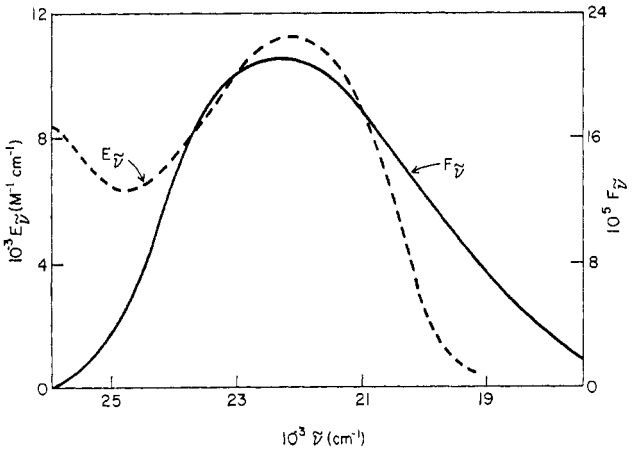

FIGURE 5: A plot of the molar extinction of FAD, $E_{\tilde{\nu}}$, and the corrected fluorescence emission spectrum of thiochrome diphosphate normalized to its total integrated spectrum, $F_{\tilde{\nu}}$, as a function of wave number, $\tilde{\nu}$. The measurements were carried out in 0.01 M potassium phosphate (pH 6.2).

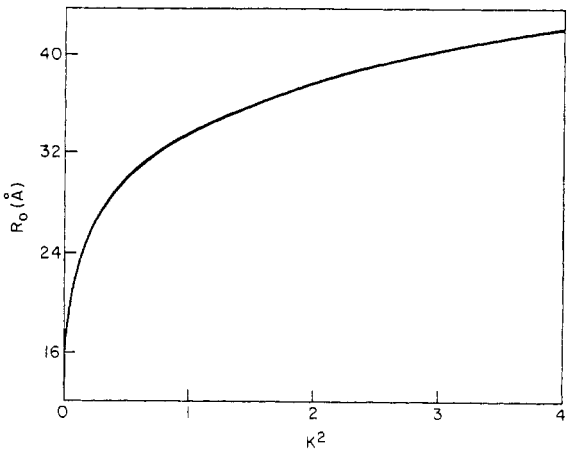

FIGURE 6: A plot of R_0, the distance at which the efficiency of fluorescence energy transfer is 0.5, *vs.* the dipole orientation factor, K^2, for the thiochrome diphosphate–FAD system. Calculations were made as described in the text using eq 7.

ments (Figure 3) preclude quantitative deductions, the qualitative agreement between these measurements and those obtained from the nanosecond fluorescence lifetime measurements is very good. Both types of experiments indicate that the quantum yield and fluorescence lifetime of thiochrome diphosphate are increased when bound to the multienzyme complex. Although this increase occurs whether or not the full complement of FAD is present on the multienzyme complex, in both experiments the measured increase in the fluorescence is slightly greater with the enzyme complex containing the smaller amount (33%) of FAD (Figures 3 and 4).

A comparison of the properties of the multienzyme complex containing only 33% FAD with those of the native enzyme (100% FAD) has shown that neither the binding of thiochrome diphosphate to the thiamine diphosphate site nor the thiamine diphosphate dependent catalytic properties of the complex are influenced by FAD removal (Moe and Hammes, 1974). In addition, experiments involving sensitive measurements of the enzyme bound FAD absorption and fluorescence spectra showed that no perturbation of these properties of the flavine accompanied the binding of various ligands to sites on the other subunit components of the multienzyme complex. The ligands tested were coenzyme A, pyruvate, guanosine triphosphate, and several substituted monophosphates. Coenzyme A binds to E_2, while the other ligands bind to E_1 (Schwartz and Reed, 1970; Shen and Atkinson, 1970). These experiments indicate that events occurring at the FAD site of the dihydrolipoyl dehydrogenase component of the complex are not detectably coupled with those occurring at the thiamine diphosphate site of the pyruvate dehydrogenase component of the complex. Thus an explanation of the observed differences in the fluorescence lifetime and intensity of thiochrome diphosphate in terms of an effect associated with FAD removal, *per se*, is unlikely. This inability to detect conformational interactions between enzymes in the complex is in marked contrast to the linked subunit conformational transitions induced by ligand binding to allosteric enzymes (*cf.* Hammes and Wu, 1974).

A mechanism involving fluorescence energy transfer between thiochrome diphosphate and FAD is a plausible and consistent explanation for the observed differences in fluorescence lifetimes. The close spectral overlap between the fluorescence emission spectrum of thiochrome diphosphate and the absorption spectrum of FAD (Figure 5) makes them an ideal donor–acceptor pair for energy transfer. (The absorption spectrum of lipoic acid also slightly overlaps the fluorescence emission spectrum of thiochrome diphosphate, but the overlap integral (eq 8) is much too small for lipoic acid to be an effective energy acceptor.)

The distance at which the efficiency of fluorescence energy transfer is one-half, R_0, can be calculated according to eq 7, except for the unknown dipole orientation factor, K^2. The value of R_0 as a function of K^2 is shown in Figure 6. The appropriate value of K^2 to use for this system is uncertain: the value of K^2 is 0.475 for an averaged orientation in a rigid medium (Steinberg, 1968) and 0.667 for the situation where averaging of the orientation occurs due to rapid rotation (relative to the fluorescence lifetime) of either the donor or acceptor. These two cases correspond to R_0 values of 30 and 31 Å, respectively. The fluorescence polarization measurements indicate that both FAD and thiochrome diphosphate have very little rotational mobility (Table I) so that the assumption of a rigid medium appears to be more appropriate. While the value of K^2 is somewhat uncertain it should be noted that R_0 does not vary greatly with K^2, except at extremely low values of K^2. For the "mid-region" where $0.02 \leq K^2 \leq 4$, the corresponding range in R_0 is 20–40 Å.

The efficiency of energy transfer can be calculated from eq 6 using the measured lifetimes. The fluorescence lifetime of free thiochrome diphosphate was determined to be 1.70 nsec, which coupled with the $\Delta\tau$ values of 0.68 and 0.81 nsec, gives fluorescence lifteimes of 2.38 and 2.51 nsec for thiochrome diphosphate bound to the multienzyme complex containing 100% FAD and 33% FAD, respectively. The extrapolated fluorescence lifetime for thiochrome diphosphate bound to the multienzyme complex containing 0% FAD is 2.58 nsec. Therefore, the efficiency of energy transfer is 0.078 and $r/R_0 = 1.51$ (eq 6). For an average orientation in a rigid medium ($K^2 = 0.475$) this corresponds to an apparent distance between energy donor and energy acceptor of 46 Å, while for averaging due to rapid rotation ($K^2 = 0.667$), the apparent distance is 47 Å. For the "mid-range" of R_0 values discussed above, the range of apparent distances is 30–60 Å.

A factor which should be considered is that a single enzyme complex contains 12 acceptors and 24 donors. Therefore, the possibility exists that a single donor can transfer energy to more than one acceptor. A more correct formulation than

eq 6 would be to express the efficiency of energy transfer from a single donor as

$$E = \sum_{i=1}^{12}(R_{0i}/r_i)^6 \Big/ \left[1 + \sum_{i=1}^{12}(R_{0i}/r_i)^6\right] \qquad (15)$$

where each donor–acceptor pair has its own characteristic R_0 due to a different orientation factor, K^2, for each pair. Unfortunately, this more general equation cannot be utilized without some knowledge of the molecular architecture of the enzyme; however the effect of multiple acceptors would be to increase further the apparent distances calculated from the measured efficiency of energy transfer. If a number of acceptors are about equidistant from the donor, an averaging of the orientation factor also might occur. The assumption has been made in the simplified analysis of the data used that one acceptor is closer to the donor than all others, and since the energy transfer falls off inversely proportional to the sixth power of the distance, only energy transfer to the closest acceptor need be considered. The validity of this approximation, which at worst provides a lower bound to the average distance between a donor and the closest acceptor not having a prohibitively small orientation factor, cannot be assessed at the present time.

The thiochrome diphosphate binds to some weaker sites on the multienzyme complex in addition to the thiamine pyrophosphate site (Moe and Hammes, 1974). Under the conditions of the energy-transfer measurements (*cf.* Figure 4), the nonspecific binding was from 6 to 7% of the total amount of thiochrome diphosphate bound. If the thiochrome diphosphate at these nonspecific sites makes some contribution to the energy transfer, the transfer between active sites would actually be less than observed, and the apparent distances calculated would be increased.

Although the distance between the thiamine diphosphate and flavine sites is uncertain because of the unknown dipole orientation factor and the multiplicity of acceptors, it very probably is close to 45 Å and the estimated minimum distance is 30 Å. This seems significantly different than the maximum distance of 28 Å predicted by the proposed catalytic mechanism involving a single rotating lipoic acid residue capable of interacting with successive active sites (Koike *et al.*, 1963). Fortunately a more precise evaluation of the distances between catalytic sites should be possible by using a variety of fluorescence probes for different specific sites. The self-consistency of the apparent distances calculated from measurements with different probes can be used to assess the range (if any) of dipole orientation factors which may be present. Such experiments are now in progress. In any event, further consideration should be given to alternative mechanisms such as substrate diffusion within the complex and acyl transfer between adjacent lipoic acid residues.

Acknowledgment

The phase fluorimeter was constructed and the lifetime measurements were carried out in the laboratories of Professor A. C. Albrecht with financial support from the National Science Foundation and the Materials Science Center of Cornell University. We gratefully acknowledge the generous assistance of Professor Albrecht.

References

Azumi, T., and McGlynn, S. P. (1962), *J. Chem. Phys. 37*, 2413.

Berlman, I. B. (1965), Handbook of Fluorescence Spectra of Aromatic Molecules, New York, N. Y., Academic Press, p 406.

Duschinsky, F. (1929), *Z. Phys. 54*, 484.

Förster, T. (1959), *Discuss. Faraday Soc. No. 27*, 7.

Hammes, G. G., and Wu, C. W. (1974), *Annu. Rev. Biophys. Bioeng 3* (in press).

Koike, M., Reed, L. J., and Carroll, W. R. (1960), *J. Biol. Chem. 235*, 1931.

Koike, M., Reed, L. J., and Carroll, W. R. (1963), *J. Biol. Chem. 238*, 30.

Moe, O. A., Jr. and Hammes, G. G. (1974), *Biochemistry 13*, 2547.

Müller, A., Lumry, R., and Kokubun, H. (1965), *Rev. Sci. Instrum. 36*, 1214.

Nawa, H., Brady, W. T., Koike, M., and Reed, L. J. (1960), *J. Amer. Chem. Soc. 82*, 896.

Parker, C. A., and Rees, W. T. (1966), *Analyst 85*, 587.

Perrin, F. (1926), *J. Phys.* [6] 7, 390.

Reed, L. J., and Mukherjee, B. B. (1969), *Methods Enzymol. 13*, 55.

Schwartz, E. R., and Reed, L. J. (1970), *Biochemistry 9*, 1434.

Shen, L. C., and Atkinson, D. E. (1970), *J. Biol. Chem. 245*, 5974.

Spencer, R. D., and Weber, G. (1970), *J. Chem. Phys. 52*, 1654.

Steinberg, I. Z. (1968), *J. Chem. Phys. 48*, 2411.

Steinberg, I. Z. (1971), *Annu. Rev. Biochem. 40*, 83.

Stryer, L., and Haugland, R. P. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 719.

Weber, G. (1966), *in* Fluorescence and Phosphorescence Analysis, Hercules, A. M., Ed., New York, N. Y., Interscience, p 222.

Willms, C. R., Oliver, R. M., Henney, H. R., Mukherjee, B. B., and Reed, L. J. (1967), *J. Biol. Chem. 242*, 889.