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Fluorescence Studies of the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli*†

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ABSTRACT: Reduced lipoic acids on the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* have been preferentially labeled by making use of the differential reactivity of lipoic acids in different environments. The lipoic acids have been labeled with *N*-ethylmaleimide, *N*-(3-pyrene)maleimide (MalPy), and *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide (DDPM). As the extent of labeling of the enzyme with MalPy increases, the fluorescence quantum yields decrease, the fluorescence polarization increases, and the emission spectrum changes in a manner indicating increased excimer formation. These results suggest that MalPy on different lipoic acids interact strongly and that some lipoic acids are very close to other lipoic acids. Energy-transfer measurements between MalPy (energy donor) and DDPM (energy acceptor) on different lipoic acids indicate that the average distance between donor and acceptor increases from 24 to 33 Å as the ratio of donors to acceptors increases. Energy-transfer measurements between MalPy (energy donor) on lipoic acids in different environments and FAD (energy acceptor) at the catalytic site of the lipoamide dehydrogenase enzyme indicate an intermolecular distance varying from 23 to >47 Å, depending on the particular lipoic acids labeled. Furthermore, the MalPy-labeled lipoic acids appear to move away from the FAD and aggregate with each other as the extent of labeling of the enzyme with MalPy increases. Energy-transfer measurements between thiochrome diphosphate (energy donor) located at the catalytic site of the pyruvate decarboxylase enzyme and DDPM (energy acceptor) labeled lipoic acids indicate little energy transfer with a variety of labeled derivatives; the intermolecular distances calculated range from 38 to >45 Å. These results are consistent with a mechanism involving multiple mobile lipoic acids which transfer acetyl groups and electrons between the three catalytic sites and adjacent lipoic acids.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* consists of three enzymes which catalyze the overall reaction (Koike et al., 1960)

$$\text{pyruvate} + \text{NAD}^+ + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (1)$$

The multienzyme complex is composed of a central core transacetylase (E_2)[1] to which a thiamine pyrophosphate requiring decarboxylase (E_1) and a lipoamide dehydrogenase flavoprotein (E_3) are bound (cf. Reed, 1974). The central core enzyme contains two covalently bound lysyllipoic acids per polypeptide chain (Danson & Perham, 1976; Shepherd & Hammes, 1977; Speckhard et al., 1977). The subunit stoichiometry of the multienzyme complex is controversial: models in which the ratio of E_1:E_2:E_3 is 24:24:12 (Reed et al., 1975) and 24–48:24:24 have been proposed (Bates et al., 1975). The molecular mechanism for the overall enzyme reaction also is not yet clear. The simplest mechanism proposed is that a single lipoic acid residue transfers the acetyl group by rotating between the three catalytic sites (Reed, 1974). This mechanism requires all of the catalytic sites to be within a radius of 14 Å. This possibility is inconsistent with resonance energy-transfer measurements: the intermolecular distances between specific binding sites and/or specific fluorescent labels indicate the catalytic sites of E_1, E_2, and E_3 and the disulfides of the lipoic acids are considerably further apart than 28 Å on the average (Shepherd & Hammes, 1976; Shepherd et al., 1976; Moe et al., 1974). However, an alternative mechanism in which multiple lipoic acids participate both to transfer the acetyl group and to oxidize the reduced lipoic acid is consistent with the distances measured (Shepherd & Hammes, 1977; Collins & Reed, 1977; Bates et al., 1977). Recent measurements have suggested that such interactions are possible and are part of the normal catalytic mechanism (Angelides & Hammes, 1978; Frey et al., 1978).

The work presented here extends previous measurements of the distances between lipoic acids and between lipoic acids and the catalytic sites on E_1 and E_3. The time course of the labeling of lipoic acid with maleimides is complex and suggests the lipoic acids within a given enzyme complex exist in several different environments (Angelides & Hammes, 1978). Because of this differential reactivity of the lipoic acids, lipoic acids in different environments can be labeled preferentially. The distances between these preferentially labeled lipoic acids and the catalytic sites of E_1 and E_3 vary significantly with the particular lipoic acids labeled. Some lipoic acids are quite close to the catalytic site of E_3 (~23 Å), some are within 38 Å of the catalytic site of E_1, and some are not very close to either (>45 Å). In addition the distance between lipoic acids in an

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[1] Abbreviations used: E_1, pyruvate decarboxylase; E_2, dihydrolipoyl transacetylase; E_3, lipoamide dehydrogenase; TPP, thiamine pyrophosphate; MalPy, *N*-(3-pyrene)maleimide; MalNEt, *N*-ethylmaleimide; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide.

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enzyme complex is within 24 Å; the close proximity of lipoic acids also is indicated by excimer formation between fluorescent labeled lipoic acids. All of the results obtained are consistent with a mechanism whereby acyl-group transfer and electron transfer involve a network of at least two lipoic acids.

Experimental Procedures

Chemicals. The MalPy was from Regis Chemical Co., DDPM, quinine bisulfate, and MalNEt were from Aldrich Chemical Co., [H^3]MalNEt was from New England Nuclear, and all other biochemicals were from Sigma. Thiochrome diphosphate was synthesized as previously described (Moe & Hammes, 1974), and its purity was checked by thin-layer chromatography in 0.15 M citrate (pH 4.0), 95% ethanol, and 1-butanol (0.6:1:0.1). Other chemicals were the best reagent grades available, and deionized–distilled water was used for all solutions.

Preparation of Pyruvate Dehydrogenase Complex and Fluorescent Maleimide Derivatives. The pyruvate dehydrogenase from *E. coli*, strain B (Miles Labs), was prepared and purified as previously described (Reed & Mukherjee, 1969). The specific activity of the enzyme complex, determined using the NAD^+ reduction assay at 30 °C (Reed & Willms, 1966), was 30–36 μmol of NADH per min per mg of protein. Protein concentrations were determined using the method of Lowry with bovine serum albumin as a standard (Lowry et al., 1951). Activities of the E_1 and E_3 components were measured using the ferricyanide and lipoamide reduction assays, respectively (Schwartz et al., 1968; Reed & Willms, 1966). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis on the enzyme complex and its derivatives was carried out as previously described (Shepherd & Hammes, 1977).

To minimize labeling of enzyme groups other than lipoic acid, the enzyme complex was incubated with nonradioactive MalNEt in the absence of substrates for 4 h at 4 °C. The reaction mixture contained 14.9 mg/mL enzyme complex, and 4.1 mM MalNEt in 20 mM potassium phosphate (pH 7.0). The enzyme treated in this way did not lose any E_1, E_3, or complex activity; after 4 h the reaction mixture was passed through a Sephadex G-25 column (25 × 2 cm i.d.) equilibrated with the reaction medium buffer at 4 °C. The enzyme was subsequently concentrated either by 45% ammonium sulfate precipitation or ultrafiltration and was finally clarified by centrifugation at 15000*g* for 20 min.

To preferentially modify the lipoic acids in different environments on E_2 with MalNEt and/or other maleimides, we took advantage of the fact that the kinetics of the TPP–pyruvate dependent maleimide incorporation proceeds in multiple phases (Angelides & Hammes, 1978). A typical reaction mixture contained 2 mg/mL of enzyme prelabeled as described above, 0.5 mM TPP, 1.9 mM pyruvate, 2.3 mM $MgCl_2$, 0.31 mM MalPy, DDPM, or MalNEt in 20 mM potassium phosphate (pH 7.0). Since maleimide stock solutions were prepared with dimethyl sulfoxide, approximately 5% dimethyl sulfoxide also was present. The reaction mixture was quenched with an aliquot of concentrated dithiothreitol (100-fold excess over the maleimide) at selected time intervals and was assayed for overall complex activity to determine the approximate amount of label incorporated. The quenched reaction mixture then was passed through a Sephadex G-25M column (PD-10), and the void volume containing the labeled enzyme complex was collected. The protein was concentrated by ultrafiltration. If desired, the enzyme could be modified with a second maleimide derivative via the same procedure. The amount of label incorporated into the enzyme was determined by radioactivity incorporation in the case of [^{3}H]MalNEt and by absorption spectroscopy in the case of MalPy and DDPM. For MalPy an extinction coefficient of 38 000 M^{-1} cm^{-1} at 342 nm in 20 mM potassium phosphate (pH 7.0) was used (Holowka & Hammes, 1977), while for DDPM an extinction coefficient of 3000 M^{-1} cm^{-1} at 440 nm in the same buffer was used (Gold & Segal, 1964).

With the labeling procedure described above, the following types of modified enzyme was prepared. (1) The lipoic acids in the more rapidly reacting environment were labeled with MalPy, a fluorescent energy donor, and the slower reacting lipoic acids were modified with DDPM, an energy acceptor. The ratio of MalPy to DDPM was varied over a wide range. (2) The order of the labeling was reversed with acceptors being incorporated first and the donors second. (3) The enzyme complex was first modified with [^{3}H]MalNEt and then with MalPy or DDPM. In this way a variety of modified enzymes can be prepared in which fluorescent labeled lipoic acids are in different environments. In conjunction with preparation of the first two types of derivatives, enzyme also was made containing only limited amounts of MalPy or DDPM.

Reduction of FAD. For many of the energy transfer measurements, the FAD was reduced in the E_3 component using sodium dithionite. The extent of reduction at 4 °C in 20 mM potassium phosphate (pH 7.0) was monitored by observing the decrease in the fluorescence emission at 520 nm with excitation at 450 nm. A 100:1 molar ratio of dithionite to FAD was sufficient to reduce the FAD under anaerobic conditions. Approximately 5% of the FAD fluorescence emission remained after this treatment. Addition of a larger molar excess of dithionite caused precipitation of the enzyme. This treatment produced some extraneous light scattering, probably due to small amounts of precipitation, which required small corrections (<5%) in the fluorescence measurements.

Spectroscopic Measurements. Ultraviolet–visible spectra were determined with a Cary 118C spectrophotometer equipped with a cell holder thermostated at 4 °C. Steady-state fluorescence excitation and emission spectra and polarization measurements were made with a Perkin-Elmer MPF3 fluorescence spectrophotometer using a microcuvette (0.3-cm path length). The fluorescence measurements were made with the sample holder thermostated at 4 °C, with dry nitrogen gas circulated throughout the sample chamber. Fluorescence spectra were corrected for wavelength-dependent variation in light-source output, phototube response, and monochromator efficiency. Corrected emission spectra were calculated from comparison of the uncorrected spectrum with that of quinine bisulfate under identical instrumental settings. The quinine bisulfate is a fluorescence standard with a known emission spectrum, and in 0.10 M H_2SO_4 was assumed to have a quantum yield of 0.7 when excited at 342 nm or 367 nm at 23 °C (Scott et al., 1970). The absorbance of the samples was kept below 0.05 (for a 1-cm path length) to minimize inner filter effects. The values of steady-state polarizations were calculated by the method of Azumi & McGlynn (1962) with excitation at 342 nm (~4-nm band width) and emission at 380 nm (12-nm band width) with appropriate corrections for the effect of the monochromator on polarization. The magnitude of light scattering of the protein derivatives was determined by measuring the apparent fluorescence emission at 360 nm with excitation at 342 nm where no significant pyrene fluorescence occurs.

The efficiency of energy transfer from MalPy–lipoic acid to DDPM–lipoic acid on the pyruvate dehydrogenase complex was measured by comparison of the fluorescence of enzyme containing only the energy donor with that of enzyme con-

taining both donor and acceptor. Both samples had identical protein concentrations (approximately 1.2 to 2.6 mg/mL). For measurements of energy transfer between MalPy–lipoic acid and FAD, the change in the fluorescence emission spectrum (342-nm excitation) upon addition of a small aliquot of concentrated dithionite was determined with appropriate corrections for the small dilution effect and the amount of unreduced FAD. In experiments where thiochrome diphosphate was used as the energy donor, energy transfer was measured by displacing the thiochrome diphosphate with TPP (final concentration, 1.0 mM). Since thiochrome diphosphate fluoresces free in solution as well as when bound to the enzyme, energy-transfer measurements were made with a 1.4 molar excess of enzyme E_1 catalytic sites over thiochrome diphosphate (about 108 μM catalytic sites, assuming the subunit stoichiometry of Reed et al., 1975) to minimize the amount of unbound thiochrome diphosphate. The fluorescence of thiochrome diphosphate is enhanced about 30% when bound to the enzyme. The fluorescence emission spectrum of the energy donor (excitation 370 nm) after displacement was compared directly with the spectrum obtained from solutions prior to displacement and with spectra obtained with unlabeled complexes. All steady-state fluorescence energy-transfer measurements were carried out in 20 mM potassium phosphate (pH 7.0) at 4 °C.

Fluorescence Lifetime Measurements. Fluorescence lifetime measurements were made with the Ortec 9200 nanosecond fluorescence spectrophotometer system interfaced to a PDP 11/20 computer (Digital Equipment Corp.). Fluorescence microcells (0.3 × 0.3 cm) were thermostated at 4 °C, and dry nitrogen gas was circulated through the sample chamber. Decay spectra for pyrene on the enzyme complex were obtained with an excitation interference filter of 340 nm (13.5-nm band-pass), and an emission interference filter of 380 nm (10-nm band-pass). The filters were obtained from Ditric Corp. Because of its size, the pyruvate dehydrogenase complex scatters light appreciably. To correct for this light scattering, a control containing unlabeled enzyme complex was treated identically to the sample, and the apparent fluorescence decay curve from these blanks was subtracted, channel by channel, from the sample decay curves. Equal protein concentrations and photon counting periods were used in both cases. Lamp spectra were collected by scattering light from a solution of Ludox (Du Pont) with the emission filter removed. The fluorescence decay curves were analyzed by deconvolution in terms of two fluorescence lifetimes by the method of moments (Isenberg & Dyson, 1969) according to the equation

$$F(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (2)$$

In eq 2, $F(t)$ is the fluorescence after correction for light scattering and the lamp pulse decay, A_1 and A_2 are amplitude parameters, τ_1 and τ_2 are fluorescence lifetimes, and t is the time. The amplitude coefficients were normalized so that $A_1 + A_2 = 1$.

Results

The modified enzyme complexes prepared are summarized in Table I. In this table the derivative is characterized by the number of lipoic acids labeled by each reagent, and the order in which the label is added. Thus the entry, 1.5 MalPy, ±40 DDPM, means the complex was first modified to give 1.5 MalPy labeled lipoic acids and then modified with DDPM to give 40 labeled lipoic acids. The ± means the enzyme complex was prepared with and without DDPM. The number of lipoic acids modified per molecule of pyruvate dehydrogenase was calculated assuming a molecular weight of 4.6 × 10^6 for the enzyme complex. Because of the uncertainty in the extinction coefficients of MalPy and DDPM and in measuring differential absorbance of the bound chromophore (especially for DDPM), the uncertainty in the stoichiometry is about 10–20%. The error is highest when the amount of label incorporated is small. The fluorescence observed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated that all of the MalPy is on E_2, and in all cases modification of the lipoic acid did not alter the enzymatic activities of E_1 and E_3 (<5% loss in activity). The specificity of the maleimide reagents for lipoic acid under the conditions utilized has been documented previously (Danson & Perham, 1976; Shepherd & Hammes, 1977).

Table I: Stoichiometry and Properties of Modified Pyruvate Dehydrogenase

derivative[a]	polarization[b]	F_{450}/F_{380}[c]
1.5 MalPy, ±40 DDPM	0.073	0.131
5 MalPy, ±35 DDPM	0.082	0.186
11 MalPy, ±30 DDPM	0.065	0.147
13 MalPy, ±26 DDPM	0.090	0.218
18 MalPy, ±20 DDPM	0.083	0.217
35 MalPy, ±7 DDPM	0.101	0.292
48 MalPy	0.126	0.283
40 MalNEt, 5 MalPy	0.086	0.059
20 MalNEt, 5 MalPy	0.071	0.067
35 DDPM, 5 MalPy		
2 DDPM		
40 MalNEt, 5 DDPM		
20 MalNEt, 2 DDPM		

[a] ± indicates the derivative was prepared both with and without DDPM. [b] Polarization of MalPy fluorescence emission at 380 nm; excitation at 342 nm, +DDPM in first six entries. [c] Ratio of MalPy fluorescence emission at 450 and 380 nm; excitation at 342 nm, +DDPM in first six entries.

The fluorescence emission spectrum of MalPy labeled lipoic acid changes significantly as the number of labeled lipoic acids per enzyme molecule changes. In Figure 1A and 1B, the emission spectra of MalPy are shown for the 1.5 MalPy derivative and the 48 MalPy derivative, respectively, with excitation at 342 nm. Unlike some cases in which pyrene maleimide apparently reacts with amino groups and results in secondary changes (Wu et al., 1976), both the excitation and emission spectra of the MalPy–transacetylase adduct remained unchanged, indicating that sulfhydryl modification is indeed the cause of the complex inactivation. The 48 MalPy–enzyme derivative shows a relatively larger amount of fluorescence at longer wavelengths; this has previously been attributed to excimer formation. Included in Table I is the ratio of fluorescence at 450 nm to that at 380 nm, F_{450}/F_{380}, for the enzyme derivatives. This is a rough measure of the amount of excimer relative to the amount of monomeric MalPy fluorescence. Also included in Table I are the polarizations of various MalPy derivatives.

Energy-transfer measurements were designed to measure several distances: the distance between lipoic acids on the same enzyme molecules with MalPy as the energy donor and DDPM the energy acceptor; the distance between MalPy (the donor)-labeled lipoic acids in different environments and FAD (the acceptor); and the distance between the catalytic site of E_1 and lipoic acids in different environments with thiochrome diphosphate as the energy donor and DDPM-labeled lipoic acid as the energy acceptor. The steady-state quantum yields for all derivatives in the presence and absence of energy acceptors are given in Table II. The fluorescence decay spectra for MalPy are well fit by eq 2. A typical decay curve in the presence and absence of DDPM for 1.5 MalPy ± 40 DDPM

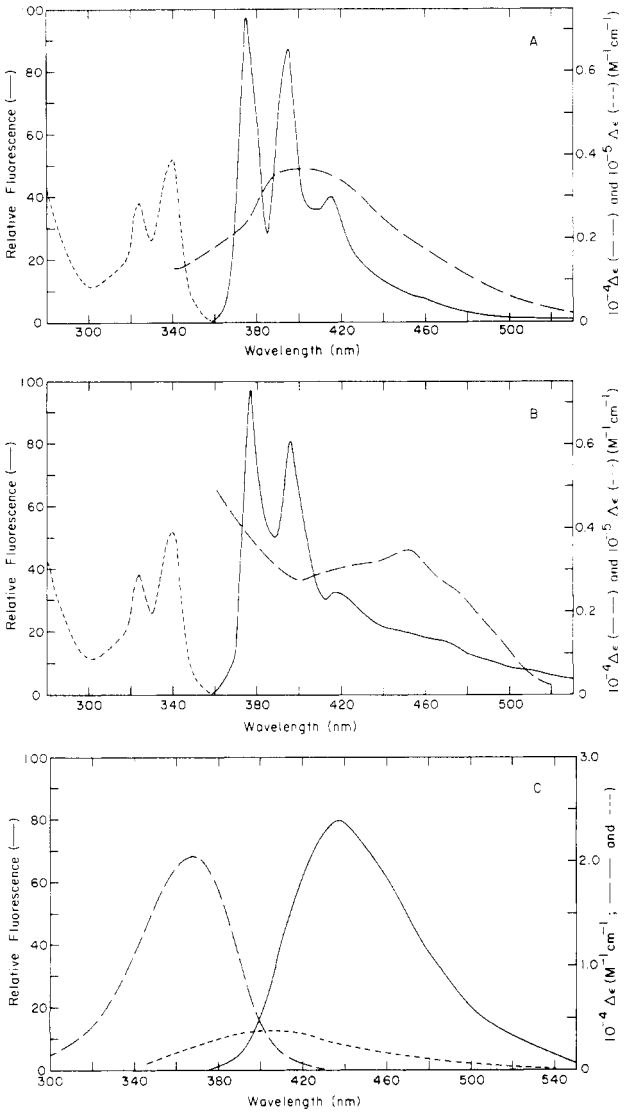

FIGURE 1: Absorption spectra and corrected fluorescence emission spectra of the MalPy and DDPM derivatives of the pyruvate dehydrogenase multienzyme complex in 20 mM potassium phosphate, pH 7.0, 4 °C. (A) Molar difference extinction coefficient $\Delta\epsilon$ (---), corrected fluorescence emission spectrum (—, 342 nm excitation, 6-nm band width) of the 1.5 MalPy–pyruvate dehydrogenase derivative, and molar difference extinction coefficient, of DDPM (— —) on the pyruvate dehydrogenase multienzyme complex. The absorption spectra were obtained from difference spectra between labeled and unlabeled enzyme. (B) Fluorescence emission spectrum (342-nm excitation, 6-nm band width) of the 48 MalPy–enzyme derivative (—) and molar difference extinction coefficient, $\Delta\epsilon$, of FAD (— —). The molar difference extinction coefficient (---) for the 48 MalPy derivative also is shown. (C) Molar difference extinction coefficient, $\Delta\epsilon$ (— —), and corrected fluorescence emission of bound thiochrome diphosphate (excitation 370 nm; —) and molar difference extinction coefficient, $\Delta\epsilon$, of the 2 DDPM–enzyme derivative (---).

is shown in Figure 2 together with the curves calculated according to eq 2. The values of A_1, A_2, τ_1, and τ_2 obtained for all derivatives are presented in Table II. Unfortunately a clear-cut theoretical link between the individual relaxation times and the amount of energy transfer is not available. Instead the results are interpreted in terms of an average relaxation time, τ_{av}:

$$\tau_{av} = A_1\tau_1 + A_2\tau_2 \tag{3}$$

This relaxation time, which is included in Table II, is proportional to the area under the fluorescence decay curve, and therefore is proportional to the quantum yield. It is important

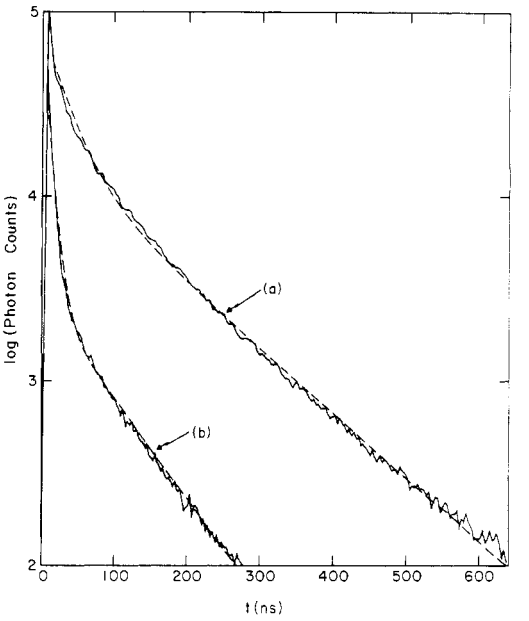

FIGURE 2: Fluorescence emission decay spectra (340-nm excitation, 380-nm emission) of 1.5 MalPy–pyruvate dehydrogenase (1.32 mg/mL) in 20 mM potassium phosphate, pH 7.0, 4 °C (a); +40 DDPM (b). The dashed lines are the reconvoluted best fit of the data to eq 2. The best fit parameters are given in Table II.

that the decay curves for the donor and donor–acceptor systems studied be measured under identical conditions (i.e., concentrations, instrument settings, etc.) to obtain meaningful comparisons. The efficiency of energy transfer can be defined as

$$E_Q = 1 - Q_{DA}/Q_D \tag{4}$$

or

$$E_\tau = 1 - \tau_{av_{DA}}/\tau_{av_D} \tag{5}$$

where E_Q is the efficiency calculated from the quantum yields in the presence and absence of the acceptor, Q_{DA} and Q_D, respectively, and E_τ is the efficiency calculated from the average fluorescence lifetimes in the presence and absence of the acceptor, $\tau_{av_{DA}}$ and τ_{av_D}, respectively. The efficiencies E_Q and E_τ are included in Table II. In the case of energy-transfer measurements involving thiochrome diphosphate and DDPM, the fluorescence lifetime of thiochrome diphosphate is too short for precise measurements so that only the steady-state value is given. The precision of energy-transfer efficiencies is such that values less than 0.05 are not significant, and even higher efficiencies are subject to an error of about ±10%. The efficiency measurements involving thiochrome diphosphate are particularly imprecise because of the presence of both free and enzyme-bound thiochrome diphosphate.

The efficiency of energy transfer for an isolated donor and acceptor is related to the distance R between them by the equation (Förster, 1966)

$$E = \frac{(R_0/R)^6}{1 + (R_0/R)^6} \tag{6}$$

where

$$R_0 = (9.79 \times 10^3)(JK^2Q_Dn^{-4})^{1/6}\text{Å} \tag{7}$$

Here J is the spectral overlap integral of the donor fluorescence emission and the acceptor absorption, K^2, is an orientation factor, Q_D is the quantum yield of the donor, and n is the refractive index of the medium. The spectral overlaps for MalPy with DDPM and FAD are shown in Figures 1A and

Table II: Energy-Transfer Parameters

donor	acceptor	Q_D	A_1	τ_1 (ns)	A_2	τ_2 (ns)	τ_{av} (ns)	E_τ	E_Q	R_0 (Å)	R (Å)
1.5 MalPy		0.272	0.817	33.8	0.183	134	52.1				
1.5 MalPy	FAD	0.213	0.826	26.7	0.174	116	42.2	0.190	0.217	31.5	40
1.5 MalPy	40 DDPM	0.067	0.937	9.88	0.063	95.5	15.3	0.706	0.754	28.5	24
5 MalPy		0.266	0.763	31.1	0.237	127	53.8				
5 MalPy	FAD	0.199	0.820	28.1	0.180	118	44.2	0.178	0.252	30.7	38
5 MalPy	35 DDPM	0.064	0.917	9.96	0.083	106	17.9	0.667	0.759	27.8	24
11 MalPy		0.256	0.799	26.2	0.201	133	47.7				
11 MalPy	FAD	0.214	0.777	22.3	0.223	111	42.1	0.117	0.164	29.6	40
11 MalPy	30 DDPM	0.141	0.886	17.6	0.114	122	29.5	0.382	0.449	27.5	29
13 MalPy		0.235	0.747	28.3	0.253	145	57.9				
13 MalPy	FAD	0.214	0.756	26.2	0.244	136	53.0	0.084	0.089	28.8	43
13 MalPy	26 DDPM	0.143	0.816	19.4	0.184	121	38.1	0.342	0.391	26.9	31
18 MalPy		0.212	0.784	27.1	0.216	132	49.8				
18 MalPy	FAD	0.201	0.789	26.3	0.211	134	49.0	0.016	0.052	28.5	>47[b]
18 MalPy	20 DDPM	0.150	0.819	16.7	0.181	123	35.9	0.279	0.292	26.4	31
35 MalPy		0.169	0.645	36.2	0.355	123	67.0				
35 MalPy	FAD	0.157	0.659	37.5	0.341	120	65.7	0.020	0.071	28.2	>46[b]
35 MalPy	7 DDPM	0.140	0.721	33.8	0.279	114	56.2	0.161	0.172	25.4	33
48 MalPy		0.134	0.812	28.1	0.188	128	46.9				
48 MalPy	FAD	0.126	0.831	26.6	0.169	132	44.5	0.051	0.060	27.8	>45[b]
40 MalNEt, 5 MalPy		0.231	0.673	31.9	0.327	133	65.0				
40 MalNEt, 5 MalPy	FAD	0.050	0.934	16.5	0.066	119	23.3	0.641	0.784	26.2	23
20 MalNEt, 5 MalPy		0.172	0.733	35.6	0.267	137	62.7				
20 MalNEt, 5 MalPy	FAD	0.101	0.757	19.3	0.243	115	42.5	0.322	0.412	26.8	29
40 MalNEt, 5 MalPy		0.266	0.563	33.7	0.437	136	78.4				
5 MalPy	35 DDPM[a]	0.0976	0.836	19.5	0.164	95.6	32.0	0.592	0.633	28.5	26
TCDP		0.245									
TCDP	2 DDPM	0.211							0.139	27.8	38
	40 MalNEt, 5 DDPM	0.244							0.004	27.8	>45[b]
	20 MalNEt, 2 DDPM	0.243							0.008	27.8	>45[b]

[a] The enzyme was first labeled with DDPM and then with MalPy. [b] Calculated assuming a maximum energy transfer efficiency of 0.05.

1B, respectively. Figure 1C shows the overlap of the corrected fluorescence emission of bound thiochrome diphosphate with the difference extinction coefficient of 2.0 mol of DDPM/enzyme complex. The spectral overlap integrals were calculated as previously described (Cantley & Hammes, 1975), and R_0 was calculated assuming $n = 1.4$ and $K^2 = {}^2/_3$, which is the value derived if the donor and acceptor rotate rapidly relative to the fluorescence lifetime. The values of R_0 obtained are given in Table II together with the values of R calculated with eq 6. In obtaining R, E_τ and E_Q were averaged; for our purposes, the error in doing this is negligible.

Discussion

The results presented here extend previous measurements of the distances between the lipoic acids on the transacetylase component and the catalytic sites on E_1 and E_3. The complexity of the time course of the labeling of lipoic acids with maleimides suggests that the lipoic acids within an enzyme complex exist in several different environments. The sensitivity of this reaction to environment is indicated by the markedly different rates of reaction with MalNEt and aromatic maleimides (Angelides & Hammes, 1978). This interpretation is supported by the different fluorescence quantum yields, emission spectra, and lifetimes of the various MalPy derivatives.

The fluorescence emission spectra of the MalPy–lipoic acid conjugates demonstrate a progressive red shift at 374 nm (~5 nm total) as the extent of labeling increases (Figure 1). The spectra have well-defined peaks around 380 and 395 nm. The emission spectrum of the 1.5 MalPy enzyme derivative exhibits better defined (i.e., sharper) peaks when compared with the spectrum obtained with all of the lipoic acids labeled. The structureless emission band at the low energy side of the spectrum centered around 450 nm (Figure 1B) is an excimer band and is discussed in more detail below. The heterogeneity of lipoic acid sites also is suggested by studies on spin-labeled lipoic acid derivatives of the intact complex (Grande et al., 1975). The fluorescence measurements clearly indicate that the lipoic acids interact. The interactions between labeled lipoic acids are manifested by changes in the fluorescence quantum yields, the lifetimes, the polarizations, and the ratio of excimer fluorescence to monomer fluorescence of the donor. The donor quantum yield decreases as the extent of donor labeling increases (Table II). Concomitant with this decrease is an increase in excimer fluorescence. This suggests that in complexes with multiple fluorescent labels, energy transfer between pyrene chromophores within the same enzyme molecule occurs. This requires the donors be quite close, probably within about 10 Å of each other (Knopp & Weber, 1969). The presence of excimer has a major effect on the quantum yield and lifetimes of the conjugates. The fluorescence polarization also tends to increase as the extent of donor labeling increases. All of these results suggest the donors interact strongly as the extent of donor labeling increases.

Both quantum yield and fluorescence lifetime measurements give similar estimates of the efficiency of energy transfer. However, the efficiencies measured by quantum yields generally are larger than those obtained with fluorescence lifetimes. This may be due to a systematic error or to the particular definition of the average lifetime used, but steady-state and lifetime techniques can measure different efficiencies if multiple donors and acceptors are present. Donor–acceptor pairs for which quenching is essentially complete would not contribute to efficiencies measured by fluorescence lifetimes but could contribute to efficiencies measured by quantum yields. In the present instance, the differences between the efficiencies calculated with steady-state

and lifetime methods are so small that the calculated values of R differ only by a few percent at most.

Several considerations are presumed in the calculation of distances between lipoic acids, between lipoic acids and FAD, and between thiochrome diphosphate and lipoic acids. The use of an average lifetime has no strict theoretical justification, and in fact the actual number of fluorescence lifetimes present is unknown. An exact treatment of this system which involves multiple transitions and multiple donors and acceptors is not possible. The implicit assumption in the treatment used is that, although multiple spectral transitions are involved, an average value of R_0 can be used. The value of R_0 depends on three parameters: the unquenched quantum yield, the spectral overlap integral, and the orientation factor K^2. Since the quantum yield is close to unity (0.13–0.27), only quantum yields close to unity contribute significantly to the overall quantum yield. If two transitions have quantum yields differing by a factor of two, a 12% difference in R_0 would result. The situation with the spectral overlap integral is similar: it should not differ greatly for the closely spaced transitions of concern, and a factor of two change in the overlap integral causes only a 12% change in R_0. The orientation factor is, of course, unknown. The validity of assuming a value of 2/3 has been discussed often (Stryer, 1978; Hillel & Wu, 1976; Matsumoto & Hammes, 1975; Dale & Eisinger, 1974). The low polarization of MalPy derivatives indicates that the use of $K^2 = 2/3$ will not be seriously in error: a *maximum* uncertainty of 20% in R can be estimated (Dale & Eisinger, 1974), and the probability distribution for R is quite narrow (Hillel & Wu, 1976). The maximum polarization of MalPy observed is 0.126. If the FAD orientation is assumed to be fixed, the probability that R has a minimum value between 36 and 48 Å is 68%; the value of R obtained with $K^2 = 2/3$ is 45 Å. This is the worst case. The minimum value of the observed MalPy polarization is 0.065. In this case, the probability that R lies between 25 and 32 Å is 90%; with $K^2 = 2/3$, $R = 27$ Å. If the acceptor is assumed to have some rotational mobility and the occurrence of multiple spectral transitions is taken into account (Haas et al., 1978), the probability distributions are even more sharply peaked. The limiting polarization of the MalPy–mercaptoethanol adduct is 0.255 (344-nm excitation, 370-nm emission). The thiochrome diphosphate polarization also is significantly less than its limiting polarization (Moe & Hammes, 1974). Thus within the context of the Förster theory and being quite pessimistic, the averaging process implicit in the data analysis is unlikely to produce an uncertainty in the calculated distances of more than ±20%.

The calculation of the distance between a donor and acceptor involves two additional considerations: the occurrence of donor–donor interactions and the presence of multiple acceptors. The presence of donor–donor interactions is indicated by excimer fluorescence. In calculating R_0, the entire emission spectrum is used in the overlap integral since the lifetimes of monomer and excimer cannot be resolved experimentally. This results in a decrease in quantum yield and overlap integral as the number of donors per enzyme molecule decreases. When multiple donors are present, the values of R calculated may be longer than the closest distance between a donor and an acceptor. This effect will, of course, be unimportant when only a small number of donors are present per enzyme molecule. The occurrence of multiple acceptors, on the other hand, causes the calculated values of R to be lower bounds to the actual distance. The effect of multiple donors and acceptors can be observed by the increase in the calculated value of the distance between lipoic acids as the ratio of donors to acceptors on lipoic acids increases, as shown in Figure 3. The calculated distance between lipoic acids varies from 24 to 32 Å during the transition from essentially all acceptors to all donors; if an average value of R is assumed (i.e., 28 Å), only a 15% deviation occurs in going from one extreme to the other.

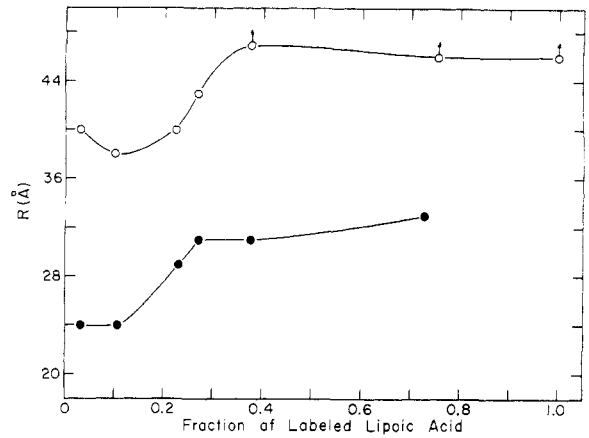

FIGURE 3: A plot of the calculated distance R vs. the fraction of MalPy labeled lipoic acids with MalPy–lipoic acid as energy donor and FAD as energy acceptor (○), and with MalPy–lipoic acid as donor and DDPM–lipoic acid as acceptor (●); ♁ indicates a lower bound. The fraction of MalPy labeled lipoic acids was calculated as the fraction of the maximum number of labels which could be incorporated into the enzyme.

The distance between labeled lipoic acid and FAD also is plotted vs. the fraction of lipoic acids labeled with MalPy for the experiments in which the amount of labeling is systematically increased (through entry twenty in Table II). The increase in distance as the fraction of labeling increases might be due to the occurrence of multiple donors, but a more likely explanation is that the lipoic acids move away from FAD and aggregate with each other; this interpretation is supported by the excimer formation and increased polarizations (Table I) as the extent of labeling with MalPy increases. The occurrence of measurable energy transfer from MalPy to FAD in *all* cases where the extent of labeling is low, while no energy transfer is seen when all of the lipoic acids are labeled with MalPy, can be attributed to this lipoic acid movement, although the occurrence of multiple donors is an alternative explanation. The experiments with a small number of MalPy labels per enzyme molecule, where donor–donor interactions and the occurrence of multiple donors can be neglected, demonstrate the nonequivalence of lipoic acid environments: different values of R are obtained for 1.5 MalPy, 20 MalNEt–5 MalPy, and 40 MalNEt–5 MalPy (Table II). These results indicate that a lipoic acid can be as close as 23 Å to FAD. The presence of multiple acceptors could influence this calculated distance to only a small extent. Only 12 flavin molecules are present per enzyme molecule (Speckhard & Frey, 1972). If two of these are equidistant from the donor, the calculated distance increases from 23 to 25 Å. If four acceptors are equidistant (which is very improbable since the diameter of the complex is about 300 Å (Reed & Cox, 1970)), the calculated distance is 29 Å. While the population of partially labeled species is necessarily heterogeneous, the self-consistent experimental results obtained over a wide range of different labeled species indicate this heterogeneity is not causing anomalous results.

A puzzling finding is that in none of the derivatives is a lipoic acid found to be within 28 Å of the thiochrome diphosphate binding site. The sensitivity of the energy-transfer experiments

involving thiochrome diphosphate is less than in the other cases, but clearly extensive energy transfer does not occur.

A mechanism for the pyruvate dehydrogenase multienzyme complex has been proposed in which at least two lipoic acids directly interact, presumably via acetyl and electron pair transfer (Angelides & Hammes, 1978; Frey et al., 1978; Collins & Reed, 1978; Bates et al., 1977). The distances between lipoic acids measured by energy transfer and suggested by excimer formation are consistent with this mechanism. The occurrence of electron transfer between flavin and lipoic acids also is consistent with the calculated distances. A cystine residue participates directly in the electron-transfer reaction in pig heart lipoamide dehydrogenase (Burleigh & Williams, 1972; Massey, 1963). The three-dimensional structure of glutathione reductase, which has an active center and mechanistic homologies with lipoamide dehydrogenase (Williams et al., 1976), has been determined (Zappe et al., 1977). The isoalloxazine ring appears to bridge the gap between the nicotinamide ring and the cystine of oxidized glutathione with the shortest distance between the nicotinamide ring and the cystine of oxidized glutathione being about 18 Å. This is similar to the shortest distance (23 Å) between FAD and MalPy–lipoic acid found with energy-transfer measurements. Furthermore, the lipoic acids appear to be able to readily change their positions and exist in different environments, as required by the proposed mechanism. The close interaction of lipoic acids with the catalytic site of E_1 remains to be demonstrated. The possibility of major conformational changes and/or the participation of a protein side chain in the acetyl group transfer to lipoic acid exists. This aspect of the catalytic mechanism is being investigated further.

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