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Rotational Diffusion Studies of the Lipoyl Domain of 2-Oxoacid Dehydrogenase Multienzyme Complexes

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ABSTRACT: Rotational mobility of the lipoyl domain of a number of 2-oxoacid dehydrogenase complexes was investigated by transient dichroism after the domain had been specifically labeled with the triplet probe eosin-5-maleimide. Complexes investigated included pyruvate dehydrogenase complexes from *Bacillus stearothermophilus*, ox heart, and *Escherichia coli* (in which the E2 component had been genetically engineered to contain one lipoyl domain) and 2-oxoglutarate dehydrogenase complexes from ox heart and *E. coli*. Measurements were also performed with ox heart pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes specifically labeled on E1. Anisotropy decays were recorded in glycerol–buffer solutions of varying viscosity and at different temperatures. For E2-labeled complexes, the decays were found to be multiexponential, and the fastest correlation time was considerably shorter than expected for tumbling of the whole complex. This fast correlation time was absent from E1-labeled complexes and was assigned to independent motion of the lipoyl domain. Plots of the fast correlation time against η/T showed a surprisingly weak dependence on viscosity and extrapolated to a time of 30–40 μs at zero viscosity. To explain this result, a model is proposed in which the lipoyl domain is in equilibrium between "free" and bound states. The time of 30–40 μs is shown to correspond to $1/k_{off}$, where k_{off} is the rate constant for dissociation of the domain from binding sites on the complex. This dissociation phenomenon only contributes to the anisotropy decay when the viscosity of the solution is sufficiently high to slow the tumbling of the whole complex to times that are long in comparison to $1/k_{off}$.

The 2-oxoacid dehydrogenase family of multienzyme complexes consists of pyruvate, 2-oxoglutarate, and branched-chain 2-oxoacid dehydrogenase complexes, which catalyze the oxidative decarboxylation of their respective substrates [for reviews, see Reed (1974) and Yeaman (1986, 1989)]. The constituent enzymes of the PDH[1] complex are pyruvate decarboxylase (E1), lipoate acetyltransferase (E2), and lipoamide dehydrogenase (E3). The corresponding enzymes of the 2-OGDH complex are 2-oxoglutarate decarboxylase (E1), lipoate succinyltransferase (E2), and lipoamide dehydrogenase (E3).

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[1] Abbreviations: PDH, pyruvate dehydrogenase; 2-OGDH, 2-oxoglutarate dehydrogenase; TPP, thiamin pyrophosphate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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In all 2-oxoacid dehydrogenase complexes [M_r = (5–10) × 10^6], the E2 component forms a structural core to which the E1 and E3 components are tightly but noncovalently attached. For the *Escherichia coli* PDH complex, E1 and E3 are arranged around a 24-unit, E2 core of octahedral symmetry, with the E1:E2 and E2:E3 binding ratios being >1.0 and <1.0, respectively. In the case of PDH complexes from *Bacillus stearothermophilus* (Henderson et al., 1979) and mammals (Reed, 1974), two nonidentical E1 components (E1α, E1β) and E3 components are assembled around a 60-unit E2 core of icosahedral symmetry, with the subunit binding ratio being approximately 0.5 (E1):1 (E2):0.5 (E3). An additional component of the mammalian PDH complex, termed component X, is involved in maximizing the catalytic efficiency of the complex as well as assisting in the binding of E3 to the E2–X subcomplex core (Powers-Greenwood et al., 1989). For both mammalian and bacterial 2-OGDH complexes the E1 and E3 component enzymes are bound to a 24-unit E2 core of octahedral symmetry.

At the heart of the reaction and common to all the 2-oxoacid dehydrogenase complexes, the lipoyllysine "swinging arm" of the E2 component allows a means of substrate transfer between the physically separated active sites of the component enzymes E1, E2, and E3. The *E. coli* PDH complex is unusual in that it contains three lipoyllysine residues per E2 component. Ox heart PDH has two lipoyl domains while the PDH complex of *B. stearothermophilus* and the 2-OGDH complexes of *E. coli* all contain only one (Yeaman, 1989).

The mode of action of the swinging arm has received much attention and was initially considered in its simplest form to be a lipoyllysine arm with a circular reach of 3 nm (Reed, 1974). However, experiments performed to measure the distances between the lipoyllysine residue and each of the individual active sites estimated the distances to be above 5 nm (Angelides & Hammes, 1979; Scouten et al., 1980), so clearly the swinging arm requires further reach. In addition, a mechanism in which the lipoyllysine swinging arm has appreciable reach, able to visit most, if not all, E1 and E3 active sites, is required to explain the phenomenon of active site coupling found within all 2-oxoacid dehydrogenase complexes (Packman et al., 1983; Berman et al., 1981; Stepp et al., 1981).

The intramolecular motion of the lipoyllysine swinging arm of 2-oxoacid dehydrogenase complexes has been studied in detail by ^{1}H NMR (Perham et al., 1981; Perham & Roberts, 1981; Wawrzynczak et al., 1981; Duckworth et al., 1982). In all spectra, sharp resonances have been detected that have been ascribed to a region of polypeptide rich in alanine or threonine residues located within the E2 component. The nucleotide sequences of all three component enzymes of *E. coli* PDH have been determined (Stephens et al., 1983a,b,c). The sequence of E2 reveals a region of polypeptide chain (20–30 amino acids in length) particularly rich in alanine and proline residues that is believed to correspond to the flexible region revealed by ^{1}H NMR (Radford et al., 1989). The structure of the E2 component is then one of two domains, an inner core forming domain, housing the transacetylase active site, linked through a region of flexible polypeptide to the outer lipoyl domain(s) which contain(s) the lipoyllysine residue. An E3 binding domain is also thought to exist, being located between the inner core forming domain and the outer lipoyl domain(s) (Packman & Perham, 1986).

In the present study, we have taken advantage of the ability to label specifically the lipoic acid of PDH complexes with maleimides (Brown & Perham, 1976). We have labeled with the triplet probe eosin-5-maleimide and employed the technique of transient dichroism (Cherry, 1978; Kinosita & Ikegami, 1988) to obtain further information on the dynamics of the lipoyl domain. Experiments were performed on PDH and 2-OGDH complexes, which we were able to label selectively in a similar manner. With the exception of ox heart PDH, we restricted our experiments to complexes whose E2 component contains only one outer lipoyl domain. PDH complexes purified from *E. coli* in which the E2 component had been genetically engineered to contain one lipoyl domain (Guest et al., 1985), from *B. stearothermophilus*, and from ox heart were studied as well as the 2-OGDH complex from both *E. coli* and ox heart.

Previous studies of the wild-type *E. coli* PDH complex by transient dichroism (Visser et al., 1981) and by electron spin resonance (Ambrose-Griffin & Griffin, 1984) have been reported.

Experimental Procedures

Materials. Purified *E. coli* [E2p(pGS110) and E2p(pGS156)] and *B. stearothermophilus* PDH complexes and *E. coli* 2-OGDH complex were kindly provided by Dr. R. N. Perham. Ox heart PDH and 2-OGDH complexes were purified essentially by the method of Stanley and Perham (1980). Eosin-5-maleimide was purchased from Molecular Probes (Eugene, OR), and *N*-ethylmaleimide was from Sigma. All other reagents and materials were of the purest grade available commercially.

Enzyme Assays. All enzyme assays were performed at 30 °C. Whole complex activities of PDH and 2-OGDH complexes were assayed by monitoring the absorbance increase of NADH at 340 nm, in the presence of the appropriate substrate, according to the method of Danson et al. (1978a). E1 activities were assayed by incubating 5–10 μg of enzyme in 1 mL of 50 mM potassium phosphate buffer, pH 7.0, containing $MgCl_2$ (1 mM), TPP (0.2 mM), dichlorophenolindophenol (72 μM), and sodium pyruvate or sodium 2-oxoglutarate (2 mM); the reaction was followed by monitoring the fall in absorbance at 600 nm. E3 activities were assayed by incubating 0.1–1 μg of enzyme in 1 mL of 50 mM sodium phosphate buffer, pH 7.6, containing NAD^+ (2.5 mM), TPP (0.2 mM), $MgCl_2$ (1 mM), and dihydrolipoamide (0.25 mM); the reaction was followed by monitoring the increase in absorbance at 340 nm.

Eosin Labeling of the Enzyme Complexes. (A) Eosin Labeling of the Lipoyllysine Residues of PDH and 2-OGDH Complexes. The procedure was based on two methods described by Brown and Perham (1976) by which 2-oxoacid dehydrogenase complexes may be specifically labeled on the lipoyllysine residue(s) of their E2 components with maleimides, (a) in the presence of substrate and (b) in the presence of NADH. Method a is as follows: PDH complex (10 mg/mL) was incubated at 4 °C in 20 mM sodium phosphate buffer, pH 7.0, containing NAD^+ (1 mM), TPP (0.2 mM), and $MgCl_2$ (2 mM). The PDH complex was equilibrated in the dark under nitrogen for 20 min, after which time whole complex, E1, and E3 activities were recorded at time zero. Eosin labeling was started by the addition of an aliquot of substrate (5 μL of sodium pyruvate, 0.1 M), mixed, and left 30 s before the addition of eosin-5-maleimide (5 μL, 5 mM). The loss of whole complex, E1, and E3 activities with time was assayed, and small aliquots (5 μL) of substrate (0.1 M) and eosin-5-maleimide (5 mM) were added at 10–15-min intervals as the rate of decrease in activity leveled off. After each addition, a further rapid decrease in activity ensued. The gradual titration of lipoyl groups by this method proved to be more effective in labeling the enzyme than a single addition of more

concentrated reagent. The reaction was stopped at 20–25% whole complex activity, by the addition of mercaptoethanol (0.2 mM), to prevent nonspecific labeling of the E1 and E3 components. Typically, E1 and E3 activities remained above 80%. Method b is as follows: 2-OGDH complex (10 mg/mL) was dialyzed overnight against 50 mM sodium phosphate, 2.7 mM EDTA, and 1 mM *N*-ethylmaleimide (NEM), pH 7.0, in order to prelabel any nonspecific labeling sites, in the absence of substrate. Prelabeling was stopped by the addition of mercaptoethanol to 0.2 mM. The reaction mixture was dialyzed for a further 3 h against labeling buffer (50 mM sodium phosphate, 2.7 mM EDTA, pH 7.0), to remove any excess NEM or mercaptoethanol. The prelabeled complex was equilibrated in the dark under nitrogen at 4 °C for 20 min. Whole enzyme, E1, and E3 activities were assayed; the whole enzyme and E1 activities had typically fallen to 10–20% of their initial values while the E3 activity remained above 80%. Eosin-5-maleimide was added to 0.3 mM, mixed, and equilibrated for 60 s before the addition of NaDH to 0.1 mM. The whole enzyme and E1 activities then fell to less than 5%. The reaction was stopped by the addition of mercaptoethanol to 0.2 mM.

The E2 components of the PDH complexes of *E. coli*, *B. stearothermophilus*, and ox heart were best specifically labeled by method a, while the 2-OGDH complexes of *E coli* and ox heart were best specifically labeled by method b.

(B) Eosin Labeling of the E1 Components of PDH and 2-OGDH Complexes. The E1 components of ox heart PDH and 2-OGDH complexes were labeled with eosin-5-maleimide. The enzyme complex (10 mg/mL) was dialyzed overnight against labeling buffer. The E1 components were specifically labeled by incubating the complex with eosin-5-maleimide (0.3 mM) for 3 h in the dark under nitrogen at 4 °C. Whole enzyme, E1, and E3 activities were recorded every 30 min; the reaction was stopped by the addition of mercaptoethanol to 0.2 mM. For ox heart PDH complex, labeling occurred without loss of whole complex, E1, or E3 activities, while for ox heart 2-OGDH complex E1 labeling was associated with loss of E1 activity.

For all eosin-labeled complexes (i.e., labeled on E1 or E2) unreacted eosin-5-maleimide was removed by passing the reaction mixture through a Sephadex G-25 gel filtration column (Pharmacia PD-10 unit, 1 × 5 cm) equilibrated with 50 mM sodium phosphate and 2.7 mM EDTA, pH 7.0. The eosin-labeled complex was then dialyzed against storage buffer (50 mM sodium phosphate, pH 7.0, 45% glycerol, 2.7 mM EDTA, 0.1 mM DTT) and stored at −20 °C.

SDS–Polyacrylamide Gel Electrophoresis. The specificity of eosin labeling was checked by SDS–PAGE according to the methods of Laemmli (1970) and Shapiro and Maizel (1969). The sites of eosin labeling on the component enzymes E1, E2, and E3 were determined by observing the positions of eosin fluorescence on the unstained slab gel, while illuminated by UV light.

Eosin:Protein Labeling Ratio. Protein concentration was determined by the method of Lowry et al. (1951) while the eosin concentration was determined with the eosin extinction coefficient at 522 nm of 8.3×10^4 M^{-1} cm^{-1} (Cherry et al., 1976). The eosin:enzyme labeling ratio was calculated from the molecular weight and component ratio of the complex of interest.

Sucrose Density Centrifugation. Eosin-labeled PDH and 2-OGDH complexes were analyzed and compared to the native unlabeled complexes by sucrose density centrifugation, with a 5–25% (w/v; 2 mL steps) linear sucrose gradient (Danson et al., 1979). The sucrose gradient was prepared in 50 mM MOPS, pH 7.0, containing 2.7 mM EDTA, 0.1 mM DTT, 0.01% Triton X-100, and 0.1 M NaCl and was centrifuged at 25000 rpm for 6 h at 20 °C. The gradient was fractionated, and protein concentrations were determined by the method of Lowry et al. (1951) and spectrophotometrically at 280 nm. Whole enzyme activity and the eosin absorbance at 531 nm were also recorded.

Transient Dichroism Measurements. The transient dichroism apparatus used to measure rotational motion was similar to that described in detail elsewhere (Cherry, 1978). However, excitation was by a Nd-YAG laser (JK Lasers, Ltd), using the frequency-doubled emission at 532 nm which is suitable for eosin probes. The pulse duration was about 15–20 ns and the repetition rate 11 Hz. Transient absorbance changes, arising from ground-state depletion, were simultaneously recorded at 515 nm for light polarized parallel and perpendicular with respect to the polarization of the exciting flash. The signals were collected and averaged by a Datalab DL 102A signal averager. Up to 1024 signals were collected in an individual experiment. Data were analyzed by calculating the absorption anisotropy $r(t)$, given by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbance changes at time t after the flash for light polarized parallel and perpendicular, respectively, with respect to the polarization of the exciting flash. $r(t)$ is independent of the signal lifetime and depends only on rotational motion, provided the absorption transient exhibits a single-exponential decay. All transient dichroism measurements were performed in 50–85% (w/v) glycerol to slow down the overall motion of the eosin-labeled complexes to the time scale of the experiment. Where possible, data were collected over two different time ranges, 0.5 and 2 ms.

Data Analysis. Since many of the anisotropy decay curves were multiexponential, a variation of global analysis (Knutson et al., 1983) was used to fit a triple exponential:

$$r(t) = \frac{r_o}{A_1 + A_2 + A_3}[A_1 \exp(-t/\varphi_1) + A_2 \exp(-t/\varphi_2) + A_3 \exp(-t/\varphi_3)] \quad (2)$$

The original principal of the global fitting technique was to use an experimental variable such as wavelength (Löfroth, 1985) or composition (Morrison et al., 1986) to produce slightly different decay profiles, which can be fitted simultaneously to the same function with different mixing coefficients. In this case, however, the only variable is data time range; the 0.5-ms data provide a higher resolution for determining φ_1, and φ_3 is obtained more accurately from the 2-ms scan. The two data sets could thus be fitted simultaneously by the same correlation times (φ_1, φ_2, and φ_3) in the same proportions (A_2:A_1 and A_3:A_1)—the global parameters—while each data set has its own individual r_o value, since this depends somewhat on experimental conditions.

Some sets of results were not suitable for this process and were fitted by a simple nonlinear least-squares program to a double-exponential decay curve. The decision to accept double- or triple-exponential decays was made by comparing values of the residual variance

$$RV = \sum_i (r_i - f_i)^2 \quad (3)$$

for a data set r_i, for the best fits f_i of the two functions. True χ^2 tests are not possible as the data are not single-photon; graphs of residuals and the autocorrelation function provided

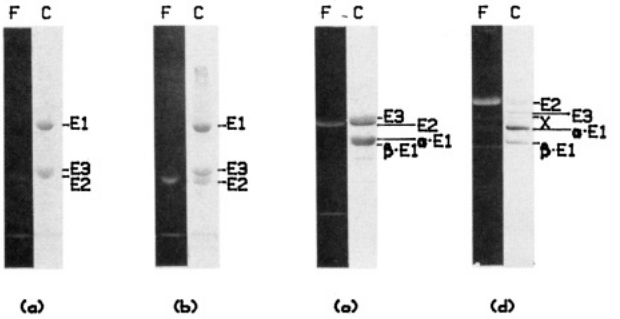

FIGURE 1: 10% SDS–PAGE of PDH complexes (F = eosin fluorescence; C = Coomassie Blue stain) specifically labeled with eosin-5-maleimide on the E2 component from (a) *E. coli* E2p(pGS156), (b) *E. coli* E2p(pGS110), (c) *B. stearothermophilus*, and (d) ox heart. Bands due to the E1, E2, E3, and (panel d only) X components are identified.

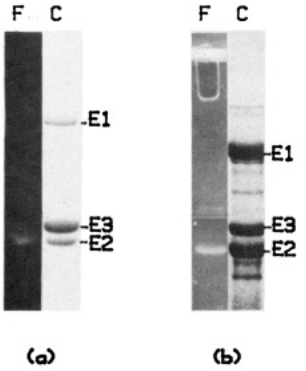

FIGURE 2: 10% SDS–PAGE of 2-OGDH complexes (F = eosin fluorescence; C = Coomassie Blue stain) specifically labeled with eosin-5-maleimide on the E2 component from (a) *E. coli* and (b) ox heart.

visual tests of absolute fit accuracy.

Results

Labeling of PDH and 2-OGDH Complexes. Figures 1 and 2 show SDS–PAGE of complexes labeled with eosin-5-maleimide in the presence of substrate or NADH. The location of the eosin probe is established by comparison of fluorescence detection with the Coomassie Blue stain detection. Figure 1 shows that both the *E. coli* [E2p(pGS110) and E2p(pGS156)] and *B. stearothermophilus* PDH complexes were labeled specifically on their E2 components, while for ox heart PDH complex a minor secondary labeling site exists on component X as reported by Lindsay et al. (1986). Figure 2 similarly shows specificity of labeling E2 components of *E. coli* and ox heart 2-OGDH complexes. The E1 components of both PDH and 2-OGDH complexes were also labeled slowly during the E2 component labeling procedure; this could be successfully eliminated by careful titration of eosin-5-maleimide into the reaction mixture and by stopping the labeling when the whole enzyme activity had been reduced to 25% of its initial activity. Both PDH and 2-OGDH complexes prelabeled with NEM could be labeled specifically with eosin in the presence of NADH on their E2 components, in the complete absence of E1 labeling. However, labeling complexes by this method caused severe aggregation of the enzyme complex as judged by low-speed centrifugation; and hence, complexes labeled in the presence of NADH were unsuitable for transient dichroism measurements. Ox heart PDH and 2-OGDH complexes were also labeled in the absence of substrate. By use of this procedure, it was found that the E1 component could be selectively labeled (Figure 3). Results of sucrose density centrifugation of ox heart PDH and 2-OGDH complexes are shown in Figure

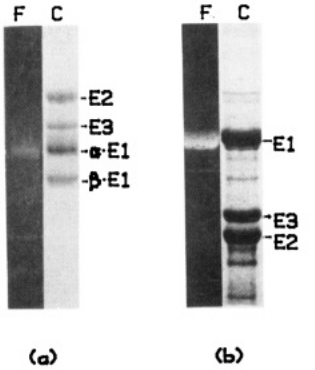

FIGURE 3: 10% SDS–PAGE of ox heart complexes (F = eosin fluorescence; C = Coomassie Blue stain) specifically labeled with eosin-5-maleimide on the E1 component: (a) PDH complex; (b) 2-OGDH complex.

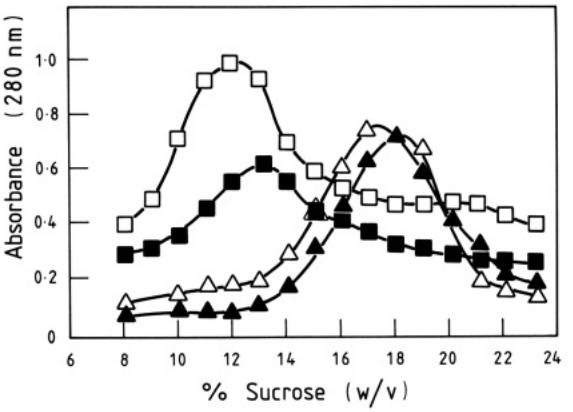

FIGURE 4: Sucrose density centrifugation of native (open symbols) and eosin-labeled (filled symbols) complexes from ox heart PDH complex labeled on the E2 subunit (triangles) and 2-OGDH complex labeled on the E1 subunit (squares).

4. Enzyme activities of unlabeled complexes were measured in solutions containing 20–80% glycerol. It was found that the complexes remained active but the rate of reaction decreased with increasing glycerol concentration.

Rotational Diffusion Measurements. Rotational diffusion of eosin–E2-labeled PDH complexes was measured at a variety of sample viscosities. The sample viscosity was varied, either with glycerol concentration (50–85%) at 2 °C or with temperature for selected glycerol concentrations.

Figure 5 shows typical anisotropy decay curves for *E. coli* E2p(pGS110) and E2p(pGS156), *B. stearothermophilus*, and ox heart eosin–E2-labeled PDH complexes. Due to limitations of material, somewhat less extensive sets of data were collected for the *E. coli* complexes compared to the other complexes. In Figure 5 and subsequent figures a few anisotropy decays are slightly displaced vertically in the interest of clarity. This has no effect on the data analysis. The data curves were fitted by triple-exponential decays as described under Experimental Procedures. The fast correlation time, φ_1, obtained from curve fitting is plotted against η/T in Figure 6.

Figure 7 shows the anisotropy decay curves for the eosin–E2-labeled *E. coli* 2-OGDH complex measured at 85 and 75% (w/v) glycerol at a temperature of 2 °C. In these cases, the anisotropy does not decay to zero but to a time-independent value, the residual anisotropy. The anisotropy decay curves were fitted to a double exponential with a residual anisotropy by global analysis.

The anisotropy decays of eosin–E1-labeled ox heart PDH and 2-OGDH complexes at 85, 65, and 50% (w/v) glycerol at 2 °C are shown in Figure 8. The anisotropy decays were fitted to a single exponential with a residual anisotropy or to

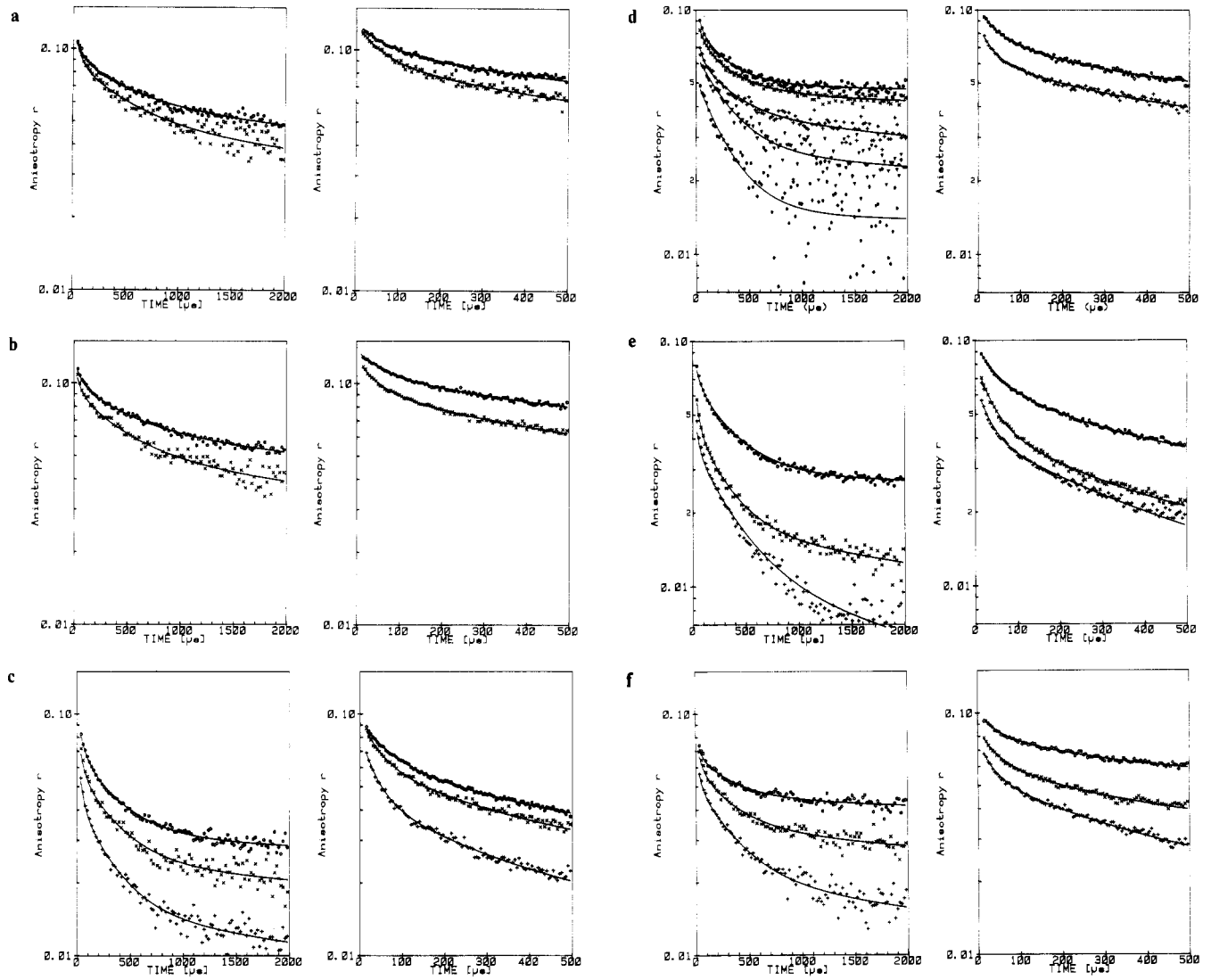

FIGURE 5: Anisotropy decay curves of PDH complexes labeled with eosin on the E2 subunit. In each case two different time scales were recorded and fitted to a triple exponential as described in the text: (a) *E. coli* [E2p(pGS110)] in 76% glycerol at 2 °C (○) and 10 °C (×); (b) *E. coli* [E2p(pGS156)] in 76% glycerol at 2 °C (○) and 10 °C (×); (c) *B. stearothermophilus* in 85% glycerol at 2 °C (○), 10 °C (×), and 20 °C (+); (d) ox heart in 85% glycerol at 2 °C (○), 10 °C (×), 20 °C (+), 30 °C (▼), and 40 °C (*); (e) *B. stearothermophilus* at 2 °C in 85% (○), 75% (×), 65% (+) glycerol; (f) ox heart at 2 °C in 85% (○), 75% (×), and 65% (+) glycerol.

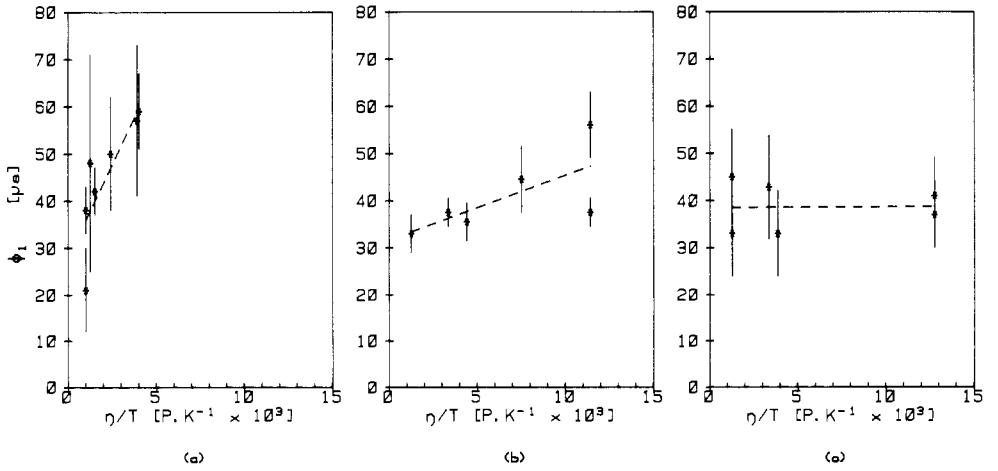

FIGURE 6: Variation of the first anisotropy decay time constant, φ_1, with viscosity and temperature, expressed as η/T, for PDH complexes from (a) *E. coli* [E2p(pGS156)], (b) *B. stearothermophilus*, and (c) ox heart. The error bars are one standard deviation, obtained from the computer fits.

a double exponential, by global analysis.

Discussion

Eosin Labeling of PDH and 2-OGDH Complexes. This study has described the specific labeling of both the E2 and E1 components of a variety of PDH and 2-OGDH complexes with eosin-5-maleimide. The conditions and sites of labeling are summarized in Table I.

Table I: Eosin Labeling Sites

	labeling conditions			
	NAD^+ + substrate	NEM prelabel	no substrate	NADH
PDH complexes				
E. coli [E2p(pGS156) and E2p(pGS110)]	E2	NI[a]	no labeling	NI
B. stearothermophilus	E2	NI	no labeling	NI
ox heart	E2 component X	E2 component X	E1α	E2 component X E1α and E1β
2-OGDH				
E. coli	E2 E3	E2	E1	NI
ox heart	E2 E1	E2	NI	E2 E1

[a] NI = not investigated.

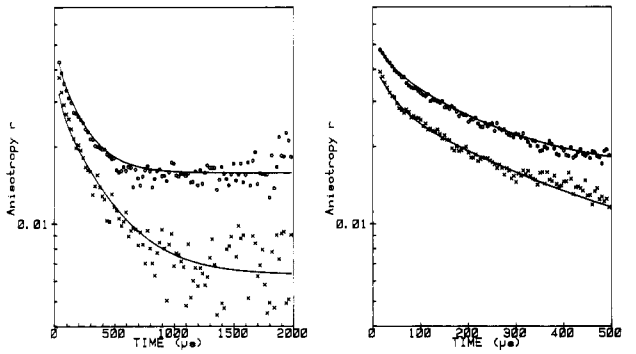

FIGURE 7: Anisotropy decay curves of the 2-OGDH complex from *E. coli* eosin labeled on the E2 subunit; two time scales were recorded and fitted globally to a double exponential and a constant term: 2 °C in 85% (O) and 75% (×) glycerol.

The expected eosin:E2 component labeling ratio is 1:1 except for ox heart PDH complex where a ratio of 2:1 is anticipated. The calculated value for ox heart 2-OGDH complex labeled in the presence of NADH correlates well with the expected ratio. However, the calculated eosin:E2 component labeling ratio for *B. stearothermophilus* PDH complex (2:1) and ox heart PDH complex (1.5:1), both labeled in the presence of substrate, and *E. coli* 2-OGDH complex (0.2:1) and ox heart PDH complex (1:1), both labeled in presence of NADH, varied significantly from the expected ratio. Possible sources of error in the calculation of these ratios include determination of the protein concentration by the method of Lowry et al. (1951) in BSA equivalents, variability in the E1:E2:E3 stoichiometry, and overestimation of the labeling ratio due to either nonspecific or noncovalent eosin labeling of the complex. Nonspecific covalent eosin labeling was minimized by NEM prelabeling in the case of the 2-OGDH complex in the absence of substrate, prior to specific eosin labeling. Prelabeling was found to be unnecessary for the PDH complexes and for 2-OGDH complexes labeled in the presence of substrate. Noncovalent eosin labeling was minimized by a combination of restrictive titration of both eosin-5-maleimide and substrate into the reaction mixture with subsequent removal of unreacted eosin-5-maleimide by an Amberlite XAD-2 or Sephadex G-25 gel filtration column. In the absence of detergent, these columns bind unreacted eosin, allowing a quick and efficient means of separating labeled complex from unreacted eosin-5-maleimide.

The eosin:E1 component labeling ratios for ox heart PDH and 2-OGDH complexes were estimated to be 1.5:1 and 6:1, respectively. Clearly, there is at least one reactive –SH group per E1 component of ox heart PDH complex and several reactive –SH groups per E1 component of ox heart 2-OGDH complex.

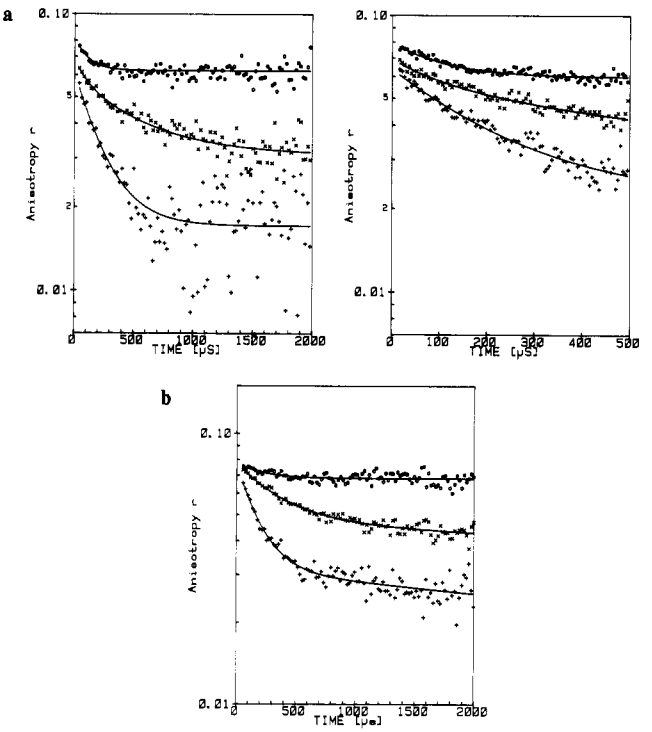

FIGURE 8: Anisotropy decay curves of 2-oxoacid dehydrogenase complexes labeled on the E1 subunit. (a) Ox heart PDH at 2 °C in 85% (O), 65% (×), and 50% (+) glycerol. Two time scales were recorded and fitted globally to a single or double exponential with a constant term. (b) Ox heart 2-OGDH at 2 °C in 85% (O), 65% (×), and 50% (+) glycerol. Due to a lack of material, only one time scale was recorded and fitted by simple least-squares analysis to a single exponential with a constant term or a double exponential.

All labeling procedures were carried out under nitrogen (where possible) and in the dark (at all times) to eliminate the possibility of photooxidative cross-linking (Kondo & Kasai, 1974) of eosin-labeled complexes. There was no evidence that any such intra- or intermolecular cross-linking occurred, as judged by SDS–PAGE and analytical centrifugation.

Transient Dichroism Measurements. The principal aim of these experiments was to study the dynamics of the lipoyl domain of the 2-oxoacid dehydrogenase complexes. Whereas fluorescence polarization measurements are only sensitive to nanosecond or faster motions, transient dichroism of triplet probes enables microsecond motions to be investigated. To observe motion in this time range, it is necessary to increase the viscosity of the solution with glycerol in order to slow the tumbling of the whole complex. Most measurements were made with the eosin probe attached to the lipoyllysine residue of E2. However, complexes labeled on E1 provided a useful

Table II: Results of Global Analysis Least-Squares Fits to Anisotropy Decay Data of Eosin-Labeled PDH and 2-OGDH Enzyme Complexes[a]

enzyme and labeling site	% glycerol	temp (°C)	φ_1 (μs) [(%)]	φ_2 (μs) [(%)]	φ_3 (ms) [(%)]
E coli [E2p(pGS156)] PDH on E2	76	1	60 ± 8 (19)	490 ± 30 (31)	9.1 (50)
E. coli [E2p(pGS110)] PDH on E2	76	1	57 ± 10 (20)	480 ± 40 (31)	9.4 (49)
B. stearothermophilus PDH on E2	65	2	33 ± 4 (40)	360 ± 20 (43)	3.5 (18)
ox heart PDH on E2	65	2	33 ± 9 (28)	350 ± 40 (44)	7 ± 2 (28)
E. coli 2-OGDH on E2	75	2	40 ± 13 (31)	343 ± 30 (54)	>20[b] (15)
ox heart PDH on E1	65	2		420 ± 30 (51)	>20[c] (49)
ox heart 2-OGDH on E1	65	2		400 ± 80 (40)	27 ± 16 (60)

[a] Error bands are computer-derived standard deviations; where none is shown, the parameter had to be held constant for convergence. [b] Fitted to a double exponential plus a constant term. [c] Fitted to a single exponential plus a constant term.

control where no contribution from the motion of the swinging arm is to be expected.

Analysis of Anisotropy Decays. At least two types of molecular motion might be expected to be associated with PDH and 2-OGDH complexes labeled with eosin on the lipoyllysine residue of their E2 components, the relatively slow tumbling of the multienzyme complex and the faster motion of the lipoyllysine swinging arm. Transient dichroism measurements were performed over two time ranges (2 and 0.5 ms) in order to resolve both types of motion. It was found that the most satisfactory fits to the anisotropy decays were obtained by global analysis in which both data sets (2 and 0.5 ms) were fitted simultaneously to the same model. For all complexes labeled on E2, either a triple-exponential decay or a double-exponential decay plus constant term gave superior fits to a double-exponential decay as judged by residual variance and by the autocorrelation of the residuals. The correlation times obtained for the different complexes are summarized in Table II.

The expected rotational correlation time φ_c for the tumbling of the whole complex may be calculated from the Stokes–Einstein equation:

$$\varphi_c = \frac{4\pi a^3 \eta}{3kT} \tag{4}$$

where a is the radius of the complex and η the viscosity of the solution. Considering the complexes to be spherical particles with diameters of 30–40 nm, the expected correlation times for the whole complex in 65% glycerol at 2 °C are calculated to be 130–300 μs (350–800 μs in 75% glycerol). From Table II, it would appear that φ_2 is of about the right magnitude to correspond to whole complex tumbling. In fact, the values of $\varphi_2 kT/\eta$ taken from Table II vary linearly with M_r as expected with the exception of ox heart 2-OGDH complexes for which the correlation time is longer than predicted.

Somewhat unexpectedly, an even slower component, $\varphi_3 >$ 1 ms, is also observed. In the case of 2-OGDH complexes this slower component is indistinguishable from a time-independent term, implying $\varphi_3 > 20$ ms. It was difficult to obtain an accurate value for φ_3, and in many cases it had to be fixed in order to attain convergence in the curve fitting. The simplest explanation of these very slow components is that they correspond to larger particles produced by complex aggregation. We were not, however, able to detect any such aggregation by analytical centrifugation. Possibly, some aggregation is induced by the glycerol concentrations used in the transient dichroism measurements.

In addition to the slow components, φ_2 and φ_3, all the E_2-labeled complexes exhibited a fast component of the anistropy decay, $\varphi_1 \sim$ 30–60 μs. This is too fast to correspond to tumbling of the whole complex. There was no evidence from analytical centrifugation to suggest that any of the complexes studied has dissociated into smaller units. It is therefore reasonable to assign this fast motion to the lipoyl domain of E_2. In confirmation of this conclusion, the fast component is absent when the eosin probe is attached to E_1 (ox heart PDH and 2-OGDH complexes). In this case, the anisotropy decays can be satisfactorily fitted either to a double-exponential decay or to a single-exponential decay plus a time-independent term. The values of φ_2 and φ_3 obtained are similar to those found for the E_2-labeled complexes.

Molecular Motion of the E_2 Lipoyl Domain. In the present study, we largely restricted our measurements to PDH and 2-OGDH complexes containing one outer lipoyl domain per E2 component, as they represent the simplest possible model of the swinging arm. The one exception, ox heart PDH complex which contains two lipoyl domains, gave results similar to those of the other complexes. The wild-type PDH complex of *E. coli* is unusual in that it contains three lipoyl domains per E2 component. However, in this case the complexes studied [E2p(pGS110) and E2p(pGS156)] had been genetically engineered (Guest et al., 1985) to contain only one lipoyl domain per E2 component, while the E2p(pGS156) PDH complex had the additional feature of the flexible polypeptide chain linking the lipoyl domain to the E2 core being shortened to roughly 65% of its native length (Graham et al., 1986). Despite these structural modifications, the specific activity and intramolecular coupling of the active sites of these complexes differed little from those of the wild-type complex (Graham et al., 1986). Taken together with the observation that the anisotropy decay curves of eosin–E2-labeled *E. coli* PDH E2p(pGS110) and E2p(pGS156) were virtually identical at all sample conditions, it suggests that the shortening of the interdomain linker region, rich in alanine and proline, does not significantly affect the molecular mobility of its lipoyl domain and hence its mechanistic action.

The sensitivity of φ_1 (the motion of the lipoyl domain) to changes in η/T was further investigated for eosin–E2-labeled PDH complexes with *E. coli* [E2p(pGS156)], *B. stearothermophilus*, and ox heart PDH complexes (Figure 6). The error bars are obtained from the curve fitting statistics and hence are an indication of the uncertainty of each individual measurement. Significantly in all cases, the fitted line (obtained by linear regression) did not pass through the origin, as would be expected for free diffusion, but intercepted the Y axis at 30–40 μs. To explain this result, we propose the following model in which for simplicity we consider only the motion of the lipoyl domain relative to an immobile complex. We suppose that there are binding sites for the lipoyl domain which anchor it to the main body of the complex. Thus, there are two different environments for the domain: (1) bound and hence immobile and (2) free, i.e., attached to E2 by the flexible arm but free to undergo independent rotational diffusion.

Let $[L_F]$ = concentration of the free lipoyl domain and $[L_B]$ = concentration of the bound lipoyl domain. At any instant in time these will be in equilibrium:

$$[L_B] \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} [L_F] \tag{5}$$

Let φ_F = rotational correlation time of the lipoyl domain. Then $\varphi_B = \varphi_F + 1/k_{off}$ will be the correlation time of the bound arm, or if $\tau_{off} = 1/k_{off}$

$$\varphi_B = \varphi_F + \tau_{off} \quad (6)$$

At the instant of the flash, there will be two components proportional to $[L_B]$ and $[L_F]$, and the observed $r(t)$ will be the sum of these, i.e.

$$r(t) = \frac{r_0}{[L_F] + [L_B] + r_\infty}\left([L_F]\exp\frac{-t}{\varphi_F} + [L_B]\exp\frac{-t}{\varphi_F + \tau_{off}} + r_\infty\right) \quad (7)$$

(r_0 is a constant to normalize the anisotropy at time zero. The term r_∞ arises because motion of the domain is likely to be over a restricted range of angles, so that domain motion does not completely randomize the probe orientation.) Now let φ_0 = correlation time for the free lipoyl domain in buffer at 20 °C. Then $\varphi_F = 293\varphi_0\eta/T$ is the correlation time in solution of viscosity η at temperature T, and eq 7 becomes

$$r(t) = \frac{r_0}{[L_F] + [L_B] + r_\infty}\left([L_F]\exp\frac{-t}{293\varphi_0\eta/T} + [L_B]\exp\frac{-t}{293\varphi_0\eta/T + \tau_{off}} + r_\infty\right) \quad (8)$$

If the M_r of the lipoyl domain is considered to be approximately 10 000, then by application of Stokes–Einstein eq 4, the correlation time (φ_0) of the free arm can be estimated to be approximately 10 ns. Hence even at η = 1000 cP, $\varphi_0\eta$ is only about 0.1 μs, and the contribution from $[L_F]$ will be too fast to be seen in experiments for which $\eta < 1000$ cP. While the value of $\varphi_0\eta$ calculated for a free domain is a minimum value, retardation of the attached domain would have to be very large to invalidate this conclusion. Thus, the contribution to $r(t)$ from the domain is likely to be

$$r(t) = \frac{r_0}{[L_F] + [L_B] + r_\infty}\left([L_B]\exp\frac{-t}{293\varphi_0\eta/T + \tau_{off}} + r_\infty\right) \quad (9)$$

For complexes in solution, this contribution will be superimposed on the tumbling of the whole complex. However, since $\varphi_2 \approx 10\varphi_1$, we can within the experimental error identify the measured value of φ_1 with the time-dependent component of eq 9, i.e.

$$\varphi_1 \approx \frac{293\varphi_0\eta}{T} + \tau_{off} \quad (10)$$

This equation is in accord with the variation of φ_1 with η/T seen in Figure 6. (Note that the slope is expected to be small due to the small value of φ_0.) The differences in the gradients of the plots of φ_1 versus η/T are probably not significant but within the relatively large experimental errors involved in measuring φ_1.

For all complexes studied, the Y intercept was between 30 and 40 μs, giving $k_{off} \approx 3 \times 10^4$ s^{-1}. If $[L_B] \gg [L_F]$, then the movement of the arm will not be detected in a low-viscosity solution since φ_B will be slower than the tumbling of the whole enzyme complex. The off-rate is fast enough to be compatible with the turnover numbers of the complexes, which are typically in the order of $(1-3) \times 10^3$ s^{-1} (Danson et al., 1978b). Detection of the motion of the lipoyl domain, in the above model, assumes that while unbound there is at least partial randomization of the absorption dipole moment of the eosin-labeled lipoyl domain. The analysis cannot give any indication of the number or spatial distribution of binding sites that the lipoyl domain can visit.

The interpretation of the present transient dichroism measurements is in excellent agreement with ^{1}H NMR studies (Roberts et al., 1982). The ^{1}H NMR spectra of 2-oxoacid dehydrogenase complexes contain a number of sharp resonances that have been assigned to a mobile region of peptide chain linking the outer lipoyl domain to the E2 core. Significantly, resonances from the amino acids in the outer lipoyl domain itself are not resolved, and intramolecular cross-linking of the outer lipoyl domain of *E. coli* PDH complex (Packman et al., 1982) had no effect on the sharp resonances. These results strongly indicate that on the NMR time scale the outer lipoyl domain remains bound to sites which restrict its motion to that of the whole complex. The value of τ_{off} of 30–40 μs deduced from the present studies is entirely consistent with this conclusion.

Previous transient dichroism measurements over a 1–10 μs time range by Visser et al. (1981) on the wild-type eosin–E2-labeled PDH complex of *E. coli* in buffer at 10 and 20 °C detected only the molecular motion associated with the tumbling of the multienzyme complex. This result is predictable from the interpretation of the present measurements since, in a low-viscosity medium, tumbling of the complex will be much faster than the off-rate of the bound lipoyl domain. Indeed, we also find that the fast component of the anisotropy decay becomes undetectable as the viscosity of the medium is decreased.

One objection to the present study is that labeling the lipoyllysine residue of E2 with eosin-5-maleimide of necessity inactivates the complexes. Scouten et al. (1980) have suggested that the eosin probe attached to the lipoyl domain also binds to the E2 core, on the basis of their failure to detect mobility in the 1–10-ns time range by fluorescence polarization. We think this is unlikely in our samples because the value of the initial anisotropy (r_0) is low (~0.08), demonstrating considerable motion of the eosin probe within the dead time of the apparatus. A low value of initial anisotropy is typical of eosin-labeled proteins and most likely results from a rapid independent motion of the probe itself (Bürkli & Cherry, 1981; Kinosita et al., 1984). Furthermore, the agreement with ^{1}H NMR data suggests that binding of the outer lipoyl domain is an intrinsic property of the complexes, not an artifact of the eosin labeling.

Taking together all investigations of the swinging arm, the weight of evidence is strongly in favor of the outer lipoyl domain spending most of its time bound, presumably at the active sites. To what extent the binding is influenced by the eosin probe or by substrate cannot be ascertained without further investigation with active complexes. In principle, this could be achieved by genetically engineering a suitable labeling site into the native complex. If this could be accomplished, further experiments along the lines of the present study should yield valuable additional information on the dynamic properties of the complexes.

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Registry No. PDH, 9014-20-4; 2-OGDH, 9031-02-1; lipoate acetyltransferase, 9032-29-5; lipoate succinyltransferase, 9032-28-4; lipoic acid, 1200-22-2.

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