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Fluorescence Polarization Studies of the Self-Association of Beef Liver Glutamate Dehydrogenase†

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ABSTRACT: Fluorescence polarization measurements on beef liver glutamate dehydrogenase conjugated with pyrenebutyric acid demonstrate that the association of this enzyme is adequately described by a reversible indefinite association with a single equilibrium constant. The data obtained with protein concentrations up to 0.7 mg/ml in 0.05 M potassium phosphate (pH 7.6) are consistent with a dissociation constant of 0.27 ± 0.02 mg/ml for an end-to-end association. The rotational relaxation time of the beef liver glutamate dehydrogenase monomer (320,000 g/mole) is 1030 ± 70 nsec at 20°. The retention of catalytic activity and sensitivity to effectors, ADP and GTP, shows that labeling causes little or no detectable change in the properties of the enzyme. Parallel experiments with dogfish glutamate dehydrogenase confirm the absence of significant association in this enzyme. Together these two cases demonstrate the usefulness of fluorescence polarization in the study of proteins undergoing self-association.

The indefinite self-association of proteins is of general interest because of its relevance to the self-assembly of biological structures and to the function of macromolecules.

Theoretical treatments of indefinite self-association with equal free energies of formation have been formulated both for ideal and nonideal cases (Van Holde and Rossetti, 1967; Adams and Lewis, 1968). Chun and Kim (1970) have presented a simplified method of graphical analysis for comparison of experimental data with the results predicted for monomer–*n*-mer and indefinite association equilibria.

Although several methods are available to obtain the thermodynamic parameters of association, sedimentation is most often applied (Roark and Yphantis, 1969; Cann, 1970) since it yields weight, number, and *z*-average molecular weights. Light scattering (Nichol *et al.*, 1964) and molecular-sieve chro-

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matography (Ackers, 1967a,b) are also used and provide weight-average molecular weights.

In this paper we describe the application of fluorescence polarization to the analysis of indefinite association equilibria with equal free energies of formation. Included is an analysis of the fluorescent conjugates of pyrenebutyric acid with glutamate dehydrogenases (EC 1.4.1.3) from beef (BL-GDH)[1] and dogfish (DF-GDH). BL-GDH undergoes end-to-end association (Sund, 1968) while DF-GDH does not (Corman *et al.*, 1967). Sedimentation (Reisler *et al.*, 1970) and light scattering (Krause *et al.*, 1970) indicate that the association of BL-GDH is of the indefinite type with a single equilibrium constant for the association.

Materials and Methods

Enzymes. BL-GDH was obtained from Sigma Chemical Co. as an ammonium sulfate suspension. DF-GDH was prepared as described by Corman *et al.* (1967) with one modification. A heat treatment, 58° for 5 min, in 5% (w/v) sodium sulfate was used in the first step.

Before experiments, the enzymes were dialyzed against 0.05 M potassium phosphate (pH 7.6) containing 10^{-4} M EDTA. The dialyzed enzymes were treated with 1 mg/ml of Norit A for 30 min and then filtered through a 0.45 μ Millipore filter (Cross and Fisher, 1970).

The concentration of BL-GDH was determined using the extinction coefficient 0.97 cm²/mg at 279 nm (Olson and Anfinsen, 1952). The DF-GDH concentration was determined using an extinction coefficient of 1.10 cm²/mg (Malencik and Anderson, 1972).

Reagents. All experiments were conducted using a 0.05 M potassium phosphate buffer (pH 7.6) containing 10^{-4} M EDTA and prepared from glass-distilled water. NADH, ADP, ATP, and GTP were obtained from Sigma Chemical Company. All other chemicals were reagent grade.

Preparation of the Fluorescent Conjugates. The mixed anhydride between pyrenebutyric acid (PBA) and sulfuric acid was prepared by the method of Rawitch *et al.* (1969); the extent of the activation reaction was followed by thin-layer chromatography (Knopp and Weber, 1967). Since the activated PBA was unstable, the activation reaction was always carried out immediately before conjugation. The concentration of the stock PBA–SO_3^- solution was 0.1 M.

Conjugation was carried out by two procedures differing only in the final concentration of PBS–SO_3^-. To prepare heavily labeled GDH (1–1.5 moles of dye/mole of protein[2]), 5 ml of GDH (8 mg/ml) in 0.05 M potassium phosphate–10^{-4} M EDTA (pH 7.6) was chilled to 0° and 0.40 ml of a 1/200 dilution of the PBA–SO_3^- in buffer was slowly added with stirring. The total time of addition was approximately 10 min. Lightly labeled GDH (0.125–0.33 mole of dye/mole of protein) was similarly prepared except that 0.6 ml of a 1/2000 dilution of PBA–SO_3^- was used. The efficiency of labeling was near 67% in the concentration range covered.

The free dye and any unreacted PBA were removed by passage through a Bio-Gel P-6 column equilibrated with buffer. The labeled protein was then dialyzed against a suspension of Dowex 2-X8 in buffer for 2 days with two changes of medium. The absorption spectra of the GDH–PBA conjugates are identical with the spectrum reported by Rawitch *et al.* (1969) for the conjugate of thyroglobulin.

The concentration of bound dye was determined from the absorption spectrum using the extinction coefficient at 346 nm, 4×10^4 cm^{-1} M^{-1} (Knopp and Weber, 1967). The protein concentration was determined by the method of Lowry *et al.* (1951).

To eliminate free dye resulting from slow hydrolysis and the reported fragments due to protease activity (Cassman and Schachman, 1971), the labeled GDH was passed through a Bio-Gel P-200 column (1 × 30 cm) immdediately before use in the experiments.

Fluorescence Measurements. All of the fluorescence polarization measurements were made using the Hitachi-Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with polarization accessory. The wavelengths of excitation and emission were 346 and 380 nm, respectively; the corresponding bandwidths were generally 2 and 4 nm. Excitation at 320, 346, and 360 nm gives similar polarizations. We selected 346 nm as the exciting wavelength in order to obtain maximum fluorescence intensity and to avoid scattering of the exciting light into the detector. The observed polarizations were corrected for partial transmission of the out of plane component by the polarizing filters. The values of T/η used in the Perrin plots were obtained by the isothermal addition of sucrose (Bingham and Jackson, 1918) and by appropriate temperature variation.

To check for scattering of the emitted light, the polarizations were measured both with and without reflecting surfaces on the two free sides of the cuvet. In the absence of scattering, the observed polarizations should be the same. Effects of stray exciting light were checked by insertion of a 350-nm cutoff filter in front of the detector and by variation of the bandwidths.

The fluorescence lifetime of the conjugates containing 0.25 mole of PBA per mole was determined at a protein concentration of 0.6 mg/ml using the monophoton fluorometer designed by Schuyler and Isenberg (1971). The total fluorescence intensity, $I_{\parallel} + 2I_{\perp}$, was measured.

Sedimentation. Sedimentation experiments were carried out with the Spinco Model E ultracentrifuge. Typically a rotor speed of 48,000 rpm and a temperature of 20° were used. The values of $s_{20,w}$ were calculated using the maximum ordinates of the gradient curves. Experiments with the scanner were conducted under the same conditions.

Assay. Rate measurements were made on a Cary 15 spectrophotometer using the 0–0.1 scale. The assay utilized the reduction of NAD^+ by glutamate under conditions described by Strecker (1955). Activation and inhibition by adenine and guanine nucleotides were compared on a relative basis.

Results

Calculations. A molecule undergoing indefinite association with the same equilibrium constant for all steps can be related to the fraction of monomer present, the dissociation constant (K), and the total protein concentration (Van Holde and Rossetti, 1967). The scheme is as follows.

$$2P_1 \underset{K}{\rightleftharpoons} P_2$$

$$P_2 + P_1 \underset{K}{\rightleftharpoons} P_3$$

$$P_{n-1} + P_1 \underset{K}{\rightleftharpoons} P_n$$

[1] Abbreviations used are: BL-GDH, glutamate dehydrogenase from beef liver; DF-GDH, glutamate dehydrogenase from dogfish liver; PBA, pyrenebutyric acid.

[2] One mole of protein is defined as 320,000 g (Cassman and Schachman, 1971).

TABLE I: Calculated Relaxation Times.

Degree of Association[a]	Symmetrical Model ρ (nsec)	Symmetrical Model $\left(1+\frac{\rho}{3\tau}\right)$	Linear Model $\frac{\rho_h}{\rho_0}$[c]	Linear Model ρ (nsec)	Linear Model $\left(1+\frac{\rho}{3\tau}\right)$
1	1030[b]	2.94	1	1030[b]	2.94
2	2060	4.88	1.1680	2406	5.53
3	3090	6.82	1.3680	4227	8.96
4	4120	8.76	1.5198	6261	12.8
5	5150	10.7	1.6234	8360	16.7
6	6180	12.6	1.6998	10,504	20.8
7	7210	14.6	1.7621	12,704	24.9
8	8240	16.5	1.7986	14,820	28.9
10	10,300	20.4	1.8769	19,332	37.4
15	15,450	30.1	1.9249	29,740	57
20	20,600	39.8	1.9531	40,233	76.8

[a] "1" refers to monomer, "2" refers to the dimer, etc. The degree of association also corresponds to the axial ratio in the linear model. [b] Value obtained from data in Figures 1 and 2. [c] Calculated by Weber (1953): personal communication, 1966.

TABLE II.[a]

	Activity Measurements of PBA–GDH Conjugates (1.5 moles of PBA/mole of protein)	
	Native GDH (%)	PBA–GDH (%)
No effector	100	100[b]
133 μM GTP	30	30
133 μM ADP	285	225
133 μM ATP	176	150

[a] Conditions: 0.05 M potassium phosphate–10^{-4} M EDTA (pH 7.6)–33 mM glutamate–330 μM NADH. [b] The specific activity of the conjugate matched that of a control sample of native GDH to which no PBA was added.

The total protein concentration, P_0, is defined as $P_0 = P_1 + 2P_2 + 3P_3 + \ldots nP_n$, where P_1, P_2, etc. represent the molar concentrations. Let $f_m, f_d, \ldots f_{n\text{-mer}}$ refer to the weight fractions of monomer, dimer, ..., and n-mer, respectively. Then $f_m = P_1/P_0$, $f_d = 2P_2/P_0 = 2f_m^2P_0/K$, $\ldots f_{n\text{-mer}} = nP_n/P_0 = nf_m^n[(P_0/K)^{n-1}]$. Since $f_m + f_d \ldots + f_n = 1$, then

$$f_m + 2f_m^2(P_0/K) + 3f_m^3[(P_0/K)^2]\ldots + nf_m^n[(P_0/K)^{n-1}] = 1 \quad (1)$$

The last equation can be solved for specified values of f_m by iteration to the desired value of n.

The principle of additivity of anisotropies (Weber, 1952) is used to relate the polarization measurements to association. The average anisotropy of a mixture of component, $\bar{A}$, equals the sum of the anisotropies of the individual components weighed by their fractional contributions to the total fluorescence intensity ($\bar{A} = \Sigma f_i A_i$). $\bar{A}$ is related to the observed polarization, p, by $\bar{A} = 1/[(1/p) - (1/3)]$. The anisotropy corresponding to the limiting polarization, p_0, is designated A_0.

The anisotropy of a specified component, A_i, is related to its rotational relaxation time (ρ_h) and excited state lifetime (τ) by the Perrin equation. $A_i = A_0/[1 + (3\tau/\rho_h)]$. Throughout these calculations we assume that τ is independent of degree of association.

Application of this information to the indefinite association model gives

$$\frac{\bar{A}}{A_0} = \frac{f_m}{\left(1+\frac{3\tau}{\rho_m}\right)} + \frac{f_d}{\left(1+\frac{3\tau}{\rho_d}\right)} \cdots + \frac{f_n}{\left(1+\frac{3\tau}{\rho_n}\right)} \quad (2)$$

where $\rho_m, \rho_d, \ldots \rho_n$ refer to the rotational relaxation times of the monomer, dimer, ..., and n-mer, respectively. These values are related to the shape of the macromolecule. Model a: For a symmetrical model, $\rho_d = 2\rho_m, \ldots \rho_n = n\rho_m$. Model b: For linear association, there is an increase in the axial ratio of one for each additional segment. Table I summarizes the values of ρ_h/ρ_0, the ratio of the mean rotational relaxation time of the prolate ellipsoid to the relaxation time of the sphere of equivalent volume, for axial ratios ranging from 1 to 20 (Weber, 1953; Weber, personal communication, 1966).

Therefore $\bar{A}$ can be calculated for both models at various fractions of association and matched with experimentally determined values. The dissociation constant (K) is then calculated from the corresponding values of P_0/K. If the indefinite association model with equal free energies of formation is applicable, the calculated K's will be independent of protein concentration.

The propagation of experimental errors can be determined by differentiation of the Perrin equation.

$$\frac{d\rho_h}{\rho_h} = \frac{\frac{dp}{p}}{\left(1-\frac{p}{p_0}\right)} \quad (3)$$

Substitution then gives

$$\frac{d\rho_h}{\rho_h} = \frac{d\rho}{p}\left[1 + \frac{1}{\frac{3\tau}{\rho_h}\left(1-\frac{p_0}{3}\right)}\right]$$

In our case, $P_0 = 0.12$. Therefore a reasonable approximation is

$$\frac{\Delta\rho_h}{\rho_h} = \frac{\Delta p}{p}\left(1+\frac{\rho_h}{3\tau}\right) \quad (4)$$

Application to Glutmate Dehydrogenase. We prepared a conjugate containing 1.5 moles of dye/mole of BL-GDH in order to check the effect of PBA labeling on the catalytic activity of the enzyme and its interactions with effectors. Table II shows the relative activities of native and conjugated GDH; Table III compares their sedimentation properties. Clearly this degree of labeling results in minimal detectable change.

TABLE III: Sedimentation Velocity Studies of BL-GDH and DF-GDH.[a]

Concentration	DF-GDH, $s_{20,w}$ (S)	BL-GDH, $s_{20,w}$ (S)
3.0 mg/ml	13,57	25.81
0.3 mg/ml	13.55	16.34
3.0 mg/ml, 1 mM NADH or NADPH, 1 mM GTP	13.2	14.50
3.0 mg/ml (1.5 moles of PBA/mole of protein)		25.23
3.0 mg/ml (1.5 moles of PBA/mole of protein), 1 mM NADH or NADPH, 1 mM GTP		15.9

[a] Conditions: 0.1 M potassium phosphate (pH 7.6), 20°, rotor speed 48,000 rpm.

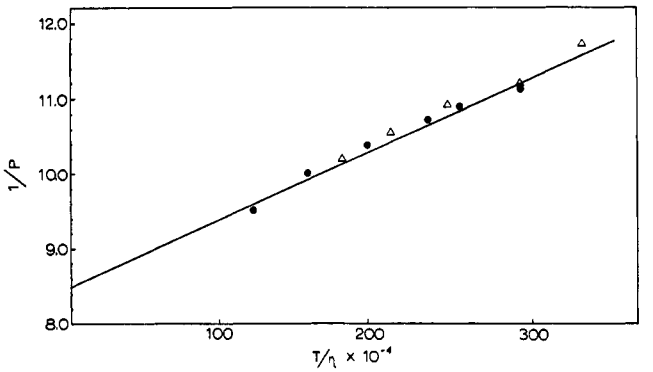

FIGURE 1: Plot of $1/p$ against T/η (degrees Kelvin/centipoise) for the conjugate of beef liver glutamate dehydrogenase containing 0.25 mole of pyrenebutyric acid/mole of enzyme. The values of T/η were obtained isothermally (20°) by addition of sucrose (●) or isotonically by temperature variation in the range 5–25° (△). Conditions: 0.05 M potassium phosphate–10^{-4} M EDTA (pH 7.6)–0.2 mg/ml of protein; λ_{ex} = 346 nm, λ_{em} = 380 nm.

The Perrin plot for BL-GDH containing 0.25 mole of PBA/mole is shown in Figure 1. The values of T/η were adjusted both by isothermal addition of sucrose and temperature variation. The agreement between the results obtained isothermally and isotonically indicates that there are no thermally activated ligand rotations—*i.e.*, the dye is rigidly attached to the protein (Wahl and Weber, 1967). The directly measured lifetime of this conjugate is 177 ± 5 nsec.[3] The limiting polarization, P_0, is independent of protein concentration and agrees with the values found by Knopp and Weber (1967) for conjugates of human macroglobulin with PBA. Similar plots were obtained with conjugates containing 0.125 and 1 mole of PBA/mole.

Dilution Experiments. Figure 2 illustrates the increase in anisotropy, $\bar{A}$, with increasing protein concentration in the range 0.02–1.2 mg/ml. The values of $\bar{A}$ were obtained by averaging 10–15 independent measurements. Concentrations above 0.1 mg/ml were generally obtained by adding native enzyme to the conjugate. Titrations using conjugate alone gave the same results obtained by adding native GDH.

The increase in anisotropy with concentration is consistent with reversible association. Extrapolation to zero protein concentration gives $\bar{A}/A_0 = 0.65 \pm 0.015$. The corresponding value of ρ is 1030 ± 70 nsec and is used as the rotational relaxation time of the monomer in the following calculations.

First we solved eq 1, both for $n = 10$ and $n = 20$, by assigning values to f_m ranging from 0.05 to 0.95 in increments of 0.05. Then we solved eq 2, using these fractions and the individual values of ρ calculated for the linear and symmetric models (Table I). The result was two sets of values of $\bar{A}$ and P_0/K (Table IV) which can be compared with experimental values. The values of P_0 in Table IV represent the protein concentrations where the observed anisotropies correspond to the calculated values. The values of K were obtained by dividing P_0 by P_0/K.

Table IV shows that the linear model fits the data reasonably throughout the concentration range 0.036–0.6 mg/ml and that the corresponding value of K is 0.27 ± 0.02 mg/ml. The symmetric model fits in the range 0.03–0.4 mg/ml and provides a value for K of 0.20 ± 0.016 mg/ml. The dashed curve in Figure 2 corresponds to the linear model with K = 0.27 mg/ml. The solid curve represents calculations for the symmetric or polydisperse model using K = 0.20 mg/ml. The two models differ significantly only at concentrations above 0.5 mg/ml. However, the standard deviation of the data points can encompass both models. Iteration to either $n = 10$ or $n = 20$ gives indistinguishable results in this concentration range.

Polarization measurements on the conjugate of DF-GDH, which is nonassociating, give $\bar{A}/A_0 = 0.72$ at a protein concentration of 0.1 mg/ml and $\bar{A}/A_0 = 0.75$ at a concentration of 1 mg/ml.

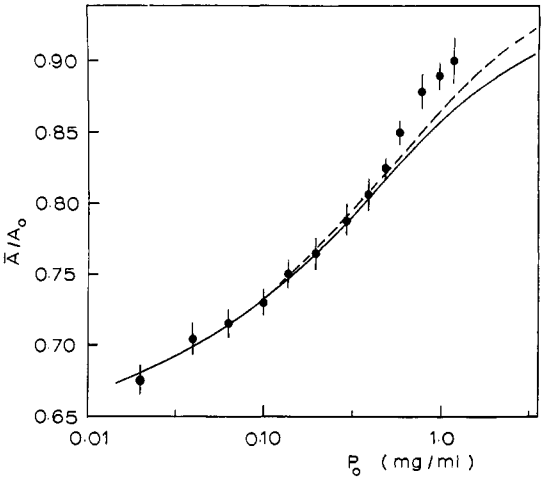

FIGURE 2: Dependence of the relative anisotropy ($\bar{A}/A_0$) on GDH concentration (P_0). Points above 0.2 mg/ml were obtained by adding native GDH to a solution containing 0.2 mg/ml of conjugate. Concentrations less than 0.2 mg/ml were obtained by dilution. The labeling varied between 0.15 and 0.4 mole of PBA/mole of protein. The range in the points encompasses the standard deviation. Calculations for an indefinite association with identical values of K using linear (– – –) and symmetric (——) models are included. The dissociation constant is 0.27 mg/ml for the linear model and 0.20 mg/ml for the symmetric model. Conditions: see Figure 1.

[3] The fluorescence time decay was consistent with a major component (*ca.* 90%) having a lifetime of 177 nsec and a minor component with a lifetime of 23 nsec.

TABLE IV: Comparison of Model Calculations with Experimental Data.

Linear Model				Symmetric Model			
$\bar{A}/A_0$	P_0/K	P_0 (mg/ml)	K (mg/ml)	$\bar{A}/A_0$	P_0/K	P_0 (mg/ml)	K (mg/ml)
0.928	15.61	1.5	0.096	0.903	15.61	1.10	0.071
0.896	6.844	1.05	0.153	0.870	6.844	0.740	0.108
0.871	4.085	0.750	0.184	0.851	4.085	0.600	0.147
0.849	2.764	0.600	0.217	0.825	2.764	0.435	0.157
0.830	2.000	0.460	0.230	0.808	2.000	0.360	0.180
0.813	1.508	0.370	0.246	0.792	1.508	0.290	0.192
0.788	0.919	0.270	0.294	0.766	0.919	0.190	0.207
0.758	0.586	0.170	0.290	0.743	0.586	0.103	0.176
0.734	0.376	0.105	0.279	0.723	0.376	0.080	0.213
0.714	0.233	0.060	0.258	0.706	0.233	0.050	0.215
0.695	0.123	0.036	0.273	0.690	0.132	0.030	0.227

Effect of Temperature. Variations in temperature between 5 and 30° have little effect on the polarization after correction to a standard value of T/η. This is consistent with the finding of Reisler and Eisenberg (1971) that the association of BL-GDH is largely entropy driven.

GTP–Coenzyme-Induced Dissociation. BL-GDH undergoes dissociation in the presence of GTP and reduced coenzyme (Frieden, 1959). In order to examine this induced dissociation, we selected two concentrations of labeled enzyme—0.15 and 1.1 mg/ml. At 0.15 mg/ml, the enzyme is largely dissociated with $s_{20,w}$ = *ca.* 14 S; at 1.1 mg/ml it is largely associated with $s_{20,w}$ = *ca.* 19 S. The addition of either coenzyme and GTP will cause dissociation to 13–14 S (Reisler *et al.*, 1970).

Figure 3 illustrates the dissociation profile using polarization measurements. The initial concentration of NADH was 10 μM. Dissociation is virtually complete after the addition of 200 μM GTP. These results are consistent with these of Huang and Frieden (1969) who followed the dissociation in absorption measurements.

Discussion

Fluorescent conjugates of beef liver glutamate dehydrogenase containing 0.15–1.5 moles of pyrenebutyric acid per mole of enzyme retain the activity, response to effectors, and sedimentation properties characteristic of the native enzyme. The activated pyrenebutyric acid probably reacts with lysine and arginine residues (Hudson, 1970). In view of the normal properties of our conjugates, preferential binding of the dye to the essential lysine residues of GDH (Anderson *et al.*, 1966; Goldin and Frieden, 1971) is unlikely.

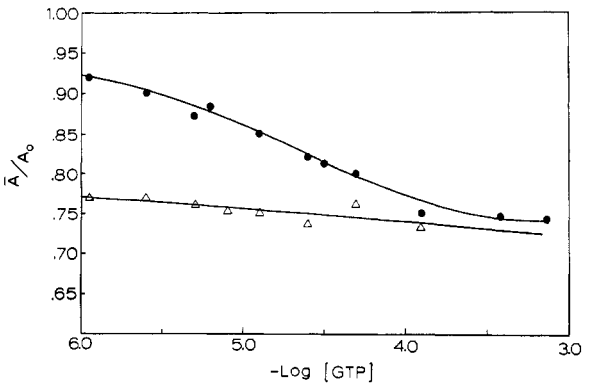

FIGURE 3: Effect of GTP addition on the relative anisotropy of BL-GDH in solutions containing 10 μM NADH. The enzyme concentrations were 0.15 mg/ml (△) and 1.1 mg/ml (●). The dye/protein ratio was 0.20. Conditions: see Figure 1.

These conjugates exhibit the long fluorescence lifetime, 177 nsec, characteristic of pyrene. Fluorescence polarization measurements conducted isothermally and isotonically show that the pyrenebutyric acid moiety is rigidly bound to the GDH molecule.

The fluorescence polarization of these conjugates increases with protein concentration. Titrations carried out either by adding unlabeled GDH to a fixed concentration of conjugate or by increasing the concentration of conjugate alone show the same concentration dependence. We have analyzed our data in terms of an indefinite association equilibrium having identical equilibrium constants for two different models. In the linear model, association of the monomers produces rigid, elongated rods. In the symmetric model, polymerization takes place without elongation.

The linear model with K = 0.27 ± 0.02 mg/ml gives an excellent fit over a 15-fold concentration range—0.036–0.6 mg/ml (Table IV). The symmetric model with K = 0.20 ± 0.016 mg/ml fits over the concentration range 0.036–0.4 mg/ml. The two models differ significantly only at concentrations above 0.5 mg/ml. However, the dissociation constant for the linear model agrees better with the value obtained under similar conditions by light scattering (Krause *et al.*, 1970). Table V compares the values of K.

If the equilibrium constant found by Krause *et al.* (1970) is applicable, we have independent evidence for the linear association of GDH. The close fit of the model shows rigid association of the monomers—*i.e.*, no appreciable internal rotations of the monomer occur in times of the order of 200 nsec or less.

Progressive deviation in K occurs at protein concentrations above *ca.* 0.6 mg/ml. Several factors may contribute to this observation. The most important of these concerns the limitation imposed on the relative magnitudes of ρ_h and τ (Brand and Witholt, 1967). The rigidly bound pyrene chromophore, with a lifetime of 177 nsec, provides information on the monomer of GDH (ρ_h = 1030 nsec) and on the lower degrees of association. However, the higher aggregates become in-

distinguishable from one another as the polarization approaches the limiting value (see eq 3 and Table I). Thus the error in K is expected to be large at high values of P_0/K.

We calculate the following distribution of species at a GDH concentration of 0.43 mg/ml using K = 0.27 mg/ml: f_1 or f_m, 0.20; f_2, 0.221; f_3, 0.183; f_4, 0.135; f_5, 0.093; f_6, 0.062; f_7, 0.04; f_8, 0.025; f_9, 0.016; and f_{10}, 0.01. Since more than 80% of the population comprises species smaller than the hexamer of molecular weight 1.9×10^6, the values of $\rho_h/3\tau$ are still favorable in the concentration range used in the calculation of K. The precision of the equilibrium constants obtained in this range, 5–10%, is comparable to that obtained from the light scattering and sedimentation measurements.

TABLE V: Comparison of Dissociation Constants for BL-GDH.

Method	K (mg/ml)
1. Sedimentation[a] (0.2 M potassium phosphate (pH 7), 20°)	0.513
2. Light scattering[b] (0.0667 M potassium phosphate (pH 7.6), 20°)	0.294
3. Fluorescence polarization (0.05 M potassium phosphate (pH 7.6), 20°)	0.27 ± 0.02 (linear model) 0.20 ± 0.016 (symmetric model)

[a] Reisler and Eisenberg, 1971. [b] Krause *et al.*, 1970.

The second factor is thermodynamic nonideality. Reisler *et al.* (1970) correct their sedimentation data for nonideality at GDH concentrations above 0.4 mg/ml. Application of nonideality corrections to fluorescence polarization has never been investigated. However, corrections are probably minimal under our conditions.

The third possibility, variation of the lifetime with association, is unlikely since the total fluorescence yield is independent of concentration.

Comparison of the rotational relaxation time of the monomer obtained from fluorescence polarization with that expected for an anhydrous sphere, ρ_0, is interesting. A value of 3.48 for ρ_h/ρ_0 is obtained. Hydration and asymmetry ordinarily account for values as large as 1.5 to 2. However, low-angle X-ray scattering indicates that the GDH molecule is loosely constructed with an effective volume 1.7 times larger than expected for a compact molecule (Pilz and Sund, 1971). Thus the open construction of the GDH molecule together with the probable hydration and asymmetry can account for the value of ρ_h/ρ_0.

Acknowledgments

We thank Dr. Robert Schuyler for the measurements of the lifetime of the excited state and Dr. E. N. Hudson for his advice on the activation of pyrenebutyric acid.

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