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Local Conformational Changes Induced by Successive Nicotinamide Adenine Dinucleotide Binding to Dissociable Tetrameric D-Glyceraldehyde-3-phosphate Dehydrogenase. Quantitative Analysis of a Two-Step Dissociation Process[†]

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ABSTRACT: Covalent binding of FITC up to 2 mol/mol of tetrameric enzyme does not affect the enzymatic activity and dissociation properties of pig muscle D-glyceraldehyde-3-phosphate dehydrogenase (GAPD). The binding of NAD to dehydrogenase–FITC complex partially reverts the quenching caused by the binding of dye to apo-GAPD. This phenomenon, as well as the formation of a characteristic absorption difference spectrum caused by the binding of NAD, makes it possible to follow the NAD-induced local conformational changes near the dye-binding region. The time course of NAD-induced spectral changes shows biphasic kinetics: a burst and a slow phase. The amplitude of burst phase as a function of NAD equivalents has sigmoidal shape due to the cooperative interaction between subunits. The same conclusion could be drawn from fluorescence anisotropy measurements. In the presence of excess NAD a slow conformational change can be detected, the amplitude of which is a function of NAD concentration. This phenomenon can be attributed to the binding of further NAD molecules to the holoenzyme. The slow phase follows first-order kinetics, and the rate constant depends on enzyme concentration. The specific fluorescence intensity and the fluorescence anisotropy of fluorescent dye labeled apo-GAPD and GAPD saturated with NAD are also dependent on enzyme concentration. We suggest that NAD binding induces major changes in the steric structure of tetrameric enzyme without influencing remarkably the interacting forces between the contact surfaces of subunits. Data are quantitatively interpreted in terms of a two-step dissociation model.

Glyceraldehyde-3-phosphate dehydrogenase (GAPD)[1] is a tetrameric enzyme composed of four chemically identical subunits (Harris & Waters, 1976). It has long been known that the mammalian enzyme exhibits negative cooperativity in the binding of NAD in the pH range 7.6–8.4, and four dissociation constants were required to describe the binding of NAD to the tetrameric enzyme (Conway & Koshland, 1968; DeVijlder & Slater, 1968; DeVijlder et al., 1969; Schlessinger & Levitzki, 1974; Bell & Dalziel, 1975; Henis & Levitzki, 1980; Price & Radda, 1971). Recently, the less cooperative binding of NAD to a special preparation of GAPD was shown (Price & Radda, 1971; Scheek & Slater, 1978; Scheek et al., 1979). Moreover, the results at pH 9.4 are consistent with independent binding of NAD at identical sites (Reynolds & Dalziel, 1979). In an assay for GAPD activity in which none of the primary products accumulate, the cooperativity for NAD tended to be positive (Aragon & Sols, 1978).

Numerous authors have also reported controversial results concerning the contribution of each of the four active sites to the gross structural and functional changes of the tetrameric enzyme upon coenzyme binding (Bell & Dalziel, 1975; Scheek & Slater, 1978; Simon, 1972; Kelemen et al., 1974; Gafni, 1981). However, in all of these studies the conformational changes of the tetrameric enzyme as a whole or of the NAD binding sites of the neighboring subunits were detected.

The association–dissociation properties of mammalian muscle apo-glyceraldehyde-3-phosphate dehydrogenase have earlier been investigated by ultracentrifugation (Hoagland &

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[1] Abbreviations: GAPD, D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); GAP, D-glyceraldehyde 3-phosphate; FITC, fluorescein isothiocyanate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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Teller, 1969; Smith & Schachman, 1973; Lakatos & Závodszky, 1976). Hoagland & Teller (1969) observed the reversible dissociation of the tetrameric dehydrogenase into dimers on dilution and excluded the existence of the trimer. Lakatos & Závodszky (1976) demonstrated the appearance of monomers on further dilution. However, quantitative data referring to the two-step dissociation of the tetrameric enzyme have not been obtained by the above method.

The tightening effect of NAD on the interactions at subunit contacts has been also intensively studied. Reynolds & Dalziel (1979) reported that combination of the last two molecules of NAD may cause eruption of the intersubunit bonds formed on binding of the first two molecules. Lakatos & Závodszky (1976) suggested from ultracentrifugation experiments that the second two tightly bound coenzyme molecules play the decisive role in the association process. The X-ray data showed that the substantial conformation changes that accompany the binding of NAD induce relatively small changes in the subunit interaction responsible for negative cooperativity (Murthy et al., 1980; Dalziel et al., 1981). On the whole the data about the effect of NAD on the association–dissociation of the enzyme are rather contradictory (Ovádi et al., 1979; Hoagland & Teller, 1969; Smith & Schachman, 1973; Osborne & Holloway, 1975; Minton & Wilf, 1981).

The aim of this paper is to give a comparative experimental analysis of the tertiary and quaternary structural changes in GAPD caused by the successive binding of NAD molecules. Moreover, a quantitative analysis of the two-step dissociation of both apoenzyme and NAD-saturated GAPD is presented. The method employed was the spectroscopic observation of a fluorescent probe, FITC, bound covalently to GAPD.

Materials and Methods

The experiments were carried out with 4 times recrystallized GAPD from pig and rabbit muscle (Elödi & Szörényi, 1956). FITC and FITC celite were from Fluka, and NAD and D-glyceraldehyde 3-phosphate were Boehringer preparations. The latter was also prepared according to Szewczuk et al. (1961). All other chemicals were reagent grade commercial preparations from Reanal (Budapest).

The concentration of the enzyme was measured spectrophotometrically by using the value $A^{0.1\%}_{280\mathrm{nm},1\mathrm{cm}} = 1.0$ (Fox & Dandliker, 1956). The molecular weight of the enzyme is 145 000 (Elödi, 1958). The specific activity of GAPD was 450–480 kat/mol of enzyme at pH 8.5 in 0.05 M Tris-HCl buffer containing 1 mM EDTA, at 20 °C. The firmly bound NAD was removed by charcoal treatment (Murdock & Koeppe, 1964). The A_{280}/A_{260} after charcoal treatment was 1.95 ± 0.05.

FITC Labeling of GAPD. Five milliters of 0.1 mM apo-GAPD in Tris-HCl or Hepes buffer containing 1 mM EDTA was treated with 2–5 mg of FITC Celite at pH 7 for 30–60 min in the dark at 4 °C. The unreacted dye was then removed by filtration of the sample through a column of Sephadex G-50 (2 × 30 cm) equilibrated with 50 mM Tris-HCl buffer and 1 mM EDTA, pH 8.5. This procedure removes all traces of noncovalently bound dye from the protein.

The dissociation of the dye from the protein upon dilution was excluded since (i) the electrophoretogram of the labeled enzyme in $NaDodSO_4$–polyacrylamide gel showed the coincidence of the protein band with the fluorescence signal and the lack of free dye (not shown) and (ii) extensive dialysis of the labeled enzyme or its rechromatography on Sephadex G-50 column did not change the degree of labeling or the values of anisotropy at the given concentrations. These data indicate that the dye binds covalently to the protein.

The concentration of labeled enzyme was determined by taking into account the absorption of FITC at 280 nm. [The free and bound dye has almost the same absorbance at 490 nm (Maeda et al., 1969).] The extent of fluorescein labeling was determined by measuring the absorbance of modified enzyme at 490 nm by using an absorption coefficient of 6.6 × 10^4 M^{-1} cm^{-1} determined by us in our experimental conditions. The degree of labeling depended on the ratio of the reactant added and the incubation time, but never exceeded 2 mol of FITC per tetramer, whether the treatment was carried out in Tris-HCl or Hepes buffer.

Spectroscopic Measurements. Fluorescence intensity and polarization were measured in a Photophysics Applied SP3 instrument by using an excitation wavelength of 470 nm and an emission wavelength of 520 nm.

For each measurement at least five determinations of the vertically (V) and horizontally (H) polarized components of the fluorescence radiation were made (standard deviation less than 5%), and the averaged background obtained for buffer solution was subtracted. The polarization (p) was calculated by using the relation $p = (V - GH)/(V + GH)$ where G is the instrumental correction according to Rank (1947). The anisotropy was calculated from the relation $A = 2p/(3 - p)$. The rotational correlation time was calculated according to the Perrin (1926) equation.

The inner filter corrected specific fluorescence intensity ($F_{\mathrm{cor}}/E_{\mathrm{T}}$, where $F_{\mathrm{cor}} = V + 2H$) was determined from the vertically (V) and horizontally (H) polarized emission intensities (Pesce et al., 1971). In some cases total fluorescence intensities were measured in an OPTON PMQ II spectrofluorometer using unpolarized light.

Absorption difference spectra were measured in special tandem cuvettes in an OPTON DMR 21 spectrophotometer. All experiments were carried out in 50 mM Tris-HCl buffer and 1 mM EDTA, pH 8.5, at 10 °C.

Gel Chromatographic Experiments. Gel chromatographic runs were made on a Sephadex G-200 column (0.6 × 96 cm) at 4 °C equilibrated with 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 1 mM NAD. The column was loaded with 1.2 mL of enzyme solution and then was eluted with buffer. A constant flow rate of 1.55 mL/h was maintained by means of an LKB Multiperpex 2115 type peristaltic pump. Effluent fractions were assayed for enzyme activity. The column was calibrated with aldolase (M_r 160 000) (Kawahara & Tanford, 1966), lactate dehydrogenase (M_r 140 000) (Jaenicke & Knof, 1968), and glycerolphosphate dehydrogenase (M_r 78 000) (Bentley et al., 1973).

The evaluation of experiments, theoretical calculations, and curve fitting were performed by the aid of a Hewlett-Packard 9810 A-type programmable calculator and plotter.

Results and Discussion

Effect of NAD Binding on the Spectroscopic Properties of Enzyme-Bound FITC. FITC as a sensitive fluorescence probe was applied to study NAD-induced changes in the steric and the quaternary structure of GAPD. Covalent binding of FITC up to 2 mol/mol of tetrameric enzyme does not influence the enzymic activity of GAPD.

Figure 1a illustrates the fluorescence spectra of the free FITC and enzyme-bound FITC in the absence and presence of NAD. In all cases the positions of the peaks of the fluorescence emission spectra excited at 470 nm are identical, but the intensities are quite different. Although the fluorescence intensity of the free dye is markedly quenched by its binding to the apoenzyme, the binding of NAD to this dehydrogenase–FITC complex partially reverts the fluorescence

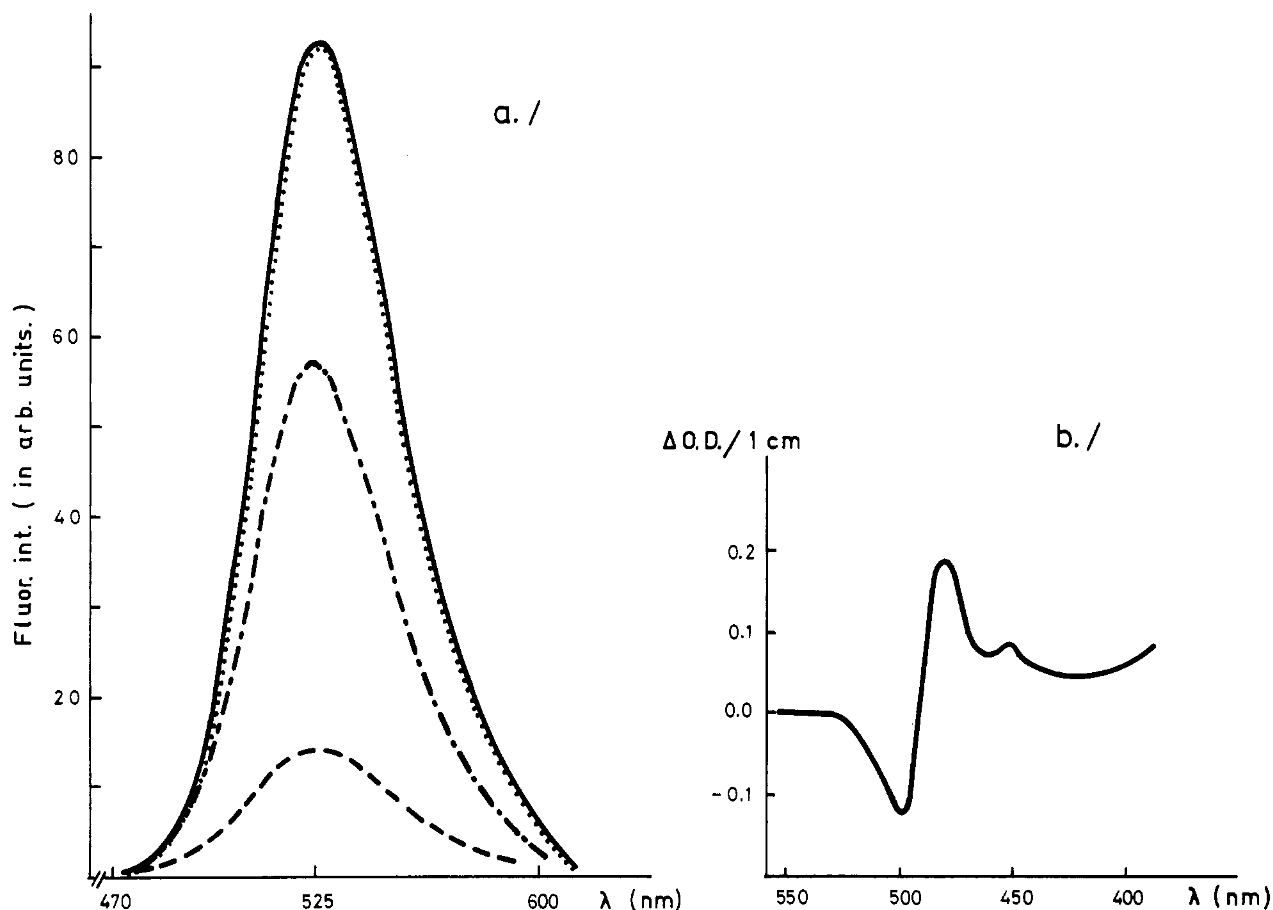

FIGURE 1: Fluorescence emission (a) of the free and GAPD-bound FITC in the presence and absence of NAD and the difference absorption spectrum (b) of the GAPD-bound FITC and NAD. In case a 8 μM FITC was excited at 470 nm, and the emission spectrum was observed in the presence (···) and absence (—) of 1 mM NAD. Apo-GAPD was treated with FITC, and the free dye was removed as described under Materials and Methods. The fluorescence emission spectrum of the GAPD-bound FITC solution having the same absorbance at 490 nm as that of the sample containing 8 μM free FITC was measured (- - -). Finally NAD at 1 mM final concentration was added to this GAPD–FITC complex and the spectrum was recorded after 30-min incubation (-··-). In case b the absorption spectrum of the mixture of GAPD at 27 μM concentration labeled covalently with 0.65 mol of FITC/mol of tetramer and 1 mM NAD was measured after 30-min incubation in tandem cuvettes against a separate solution of the labeled enzyme and NAD at 10 °C.

intensity. Control experiments showed that the fluorescence intensity of the free FITC is not affected by NAD.

The fact that the fluorescence intensity of free FITC decreased considerably on binding to the apoenzyme can be attributed, in addition to the covalent linkage, to hydrophobic interactions between the enzyme surface and the dye molecule (Bentley et al., 1973). However, some of these hydrophobic forces can be abolished as a consequence of the binding of NAD to FITC-labeled apo-GAPD, whereby the dye becomes more fluorescent.

A similar phenomenon can be observed by measuring the effect of NAD on the absorption properties of apoenzyme–FITC complex. Figure 1b shows the absorption difference spectrum of FITC–enzyme–NAD and the labeled enzyme and NAD, measured after it had reached maximum amplitude. In the visible region a sharp difference maximum and a minimum can be observed at 480 and 500 nm, respectively. The molar extinction coefficient calculated from the difference maximum is 1.1×10^4 M^{-1} cm^{-1}. Since the maximum of the absorption spectrum of free FITC is at 490 nm, the formation of an absorption difference spectrum may be the indication of NAD-induced local conformational changes in the FITC binding region.

Kinetics of the NAD-Induced Conformational Changes. The time course of the formation of the difference spectrum of FITC-labeled GAPD due to the binding of NAD is biphasic with a burst and a slow phase (Figure 2).

The burst monitored at 480 nm developed immediately with the addition of stoichiometric amounts of NAD. Figure 3 shows the sigmoidal shape of the absorbance change at 480 nm and of the fluorescence anisotropies. The shape was independent of the amount of FITC bound per tetramer, up to 2 equiv. Control experiments showed that in the same conditions the formation of the Racker band (Racker & Krimsky, 1952) was not affected by FITC. Thus the NAD-induced conformational changes are reflected in the dye's environment. Since the subunits are only partially labeled with dye, the sigmoidal character of the burst phases as a consequence of the addition of NAD must be the result of subunit interactions.

A similar conclusion can be drawn from the anisotropy change. However, in this case the changes reflect the conformational alterations of the tetrameric enzyme as a whole over the local conformational changes near the dye binding site. Thus the dramatic decrease of the anisotropy values during the NAD saturation—when the rotational correlation time calculated by Perrin (Perrin, 1926) changes from 24 to 4 ns (Table I)—can be attributed to the tetrameric structure of GAPD becoming more compact, and/or the enzyme-bound FITC becoming more flexible. It should be noted that the rotational correlation time (24 ns) determined for the tetrameric apoenzyme from the measured anisotropy value (A = 0.33 in Figure 3) is about half of that calculated for a tetramer having a molecular weight of 145 000, and the dye binds completely rigidly (56 ns). This difference means that

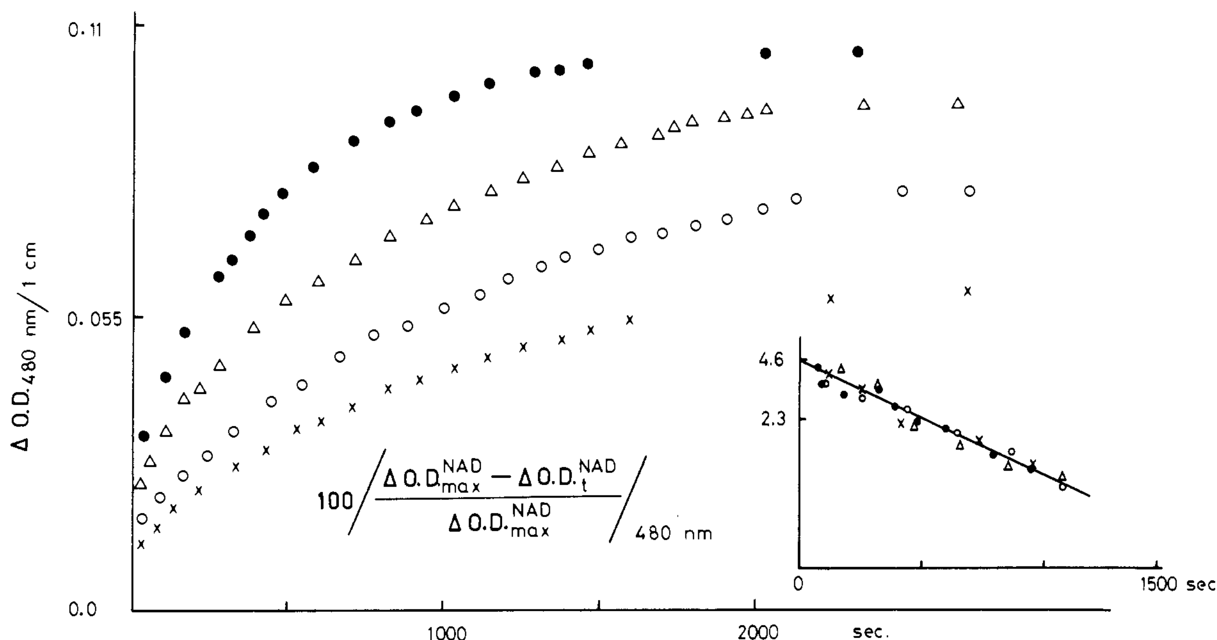

FIGURE 2: Kinetics of the formation of the difference absorption of the FITC-labeled GAPD and NAD at 480 nm. The NAD concentrations were the following: 60 μM (×), 120 μM (○), 150 μM (△), and 0.3–2 mM (●). Concentration of GAPD was 13 μM containing 0.7 mol of FITC/mol of tetramer. For other conditions see Figure 1b. Insert shows the semilogarithmic plot of the first-order reaction of the slow phase following the burst. ΔOD^{NAD}_{max} is the limit value determined at the actual NAD concentrations.

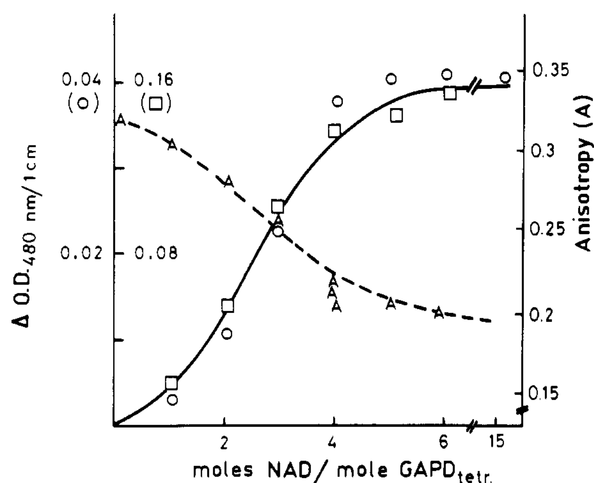

FIGURE 3: NAD concentration dependence of the extent of the burst of the kinetics presented in Figure 2 (○ and □) and of fluorescence anisotropy (A). The concentration of GAPD was 27 μM in all experiments, but the covalently labeled FITC was 0.5 (○) and 18.5 mol/mol of tetramer (□ and A). All conditions were the same as in Figure 1, except that the absorption was measured after 1-min incubation.

the dye has an individual freedom for rotation on the enzyme surface which during NAD saturation becomes more flexible and consequently more fluorescent.

After the burst reaction a further slow elevation of the absorbance at 480 nm was observed as shown in Figure 2. This slow phase follows first-order kinetics. The rate constant $k = 1.3 \times 10^{-3}$ s^{-1}, measured at 1.9 μM enzyme concentration, is independent of NAD concentration (Figure 2, inset). However, the amplitude of ΔOD_{480} increases with NAD concentration, and it reaches a limit value over 0.3 mM NAD. These results indicate that the slow phase reflects further conformational changes induced by the additional binding of NAD. The increase of the amplitude as a function of NAD concentration over the stoichiometric amount suggests the existence of NAD binding sites outside the active center as it was proposed also previously (Batke & Keleti, 1968; Sapag-Hagar, 1969; Ovádi et al., 1972). We have observed that in the protein concentration range tested, the molar extinction coefficient of ΔOD_{480} of the difference spectrum caused by NAD binding to FITC-labeled dehydrogenase was independent of enzyme concentration, if NAD concentration was 1 mM or higher. This finding may indicate that in these conditions either all of the different oligomeric forms are saturated with NAD or only one type of oligomer is present in this system. However, the kinetics of the slow phase at different enzyme concentrations in the presence of excess of NAD follows first-order kinetics, but the apparent rate constant depends on enzyme concentration (Figure 4). This phenomenon can be explained by assuming the existence of different oligomeric forms with different abilities to tolerate the NAD-induced conformational changes.

Table I: Rotational Correlation Time (ρ) of Different Forms of GAPD[a]

ρ calculated according to	apoenzyme (ns) tetramer	apoenzyme (ns) monomer	holoenzyme (ns) tetramer	holoenzyme (ns) monomer
$\rho = A\tau/(A_0 - A)$	24	0.26	4	0.26
$\rho = M_w(\bar{v} + h)\eta/(RT)$	56	14	56	14

[a] The calculations were carried out according to the Perrin equation (Perrin, 1926). $A_0 = 0.4$; $\tau = 5$ ns; M_w 145 000; $\eta = 0.01$ g/(s·cm); $\bar{v} = 0.73$ mL/g; $h = 0.2$ mL/g; T = 293 K. No difference of the lifetime (τ) of FITC was assumed in its unbound and enzyme-bound forms.

Quantitative Evaluation of the Two-Step Dissociation of Apo-GAPD. A stock solution of apo-GAPD (0.1 mM) labeled covalently by FITC was diluted with the standard Tris-HCl buffer, pH 8.5, step by step, and the anistropy values at different concentrations were determined after the anisotropy had reached a constant value. Figure 5 shows the enzyme-concentration dependence of the anistropy values corresponding to the final equilibrium state (A_{eq}). We also measured the fluorescence intensity in each sample and calculated the corrected specific fluorescence intensity. Both the anisotropy

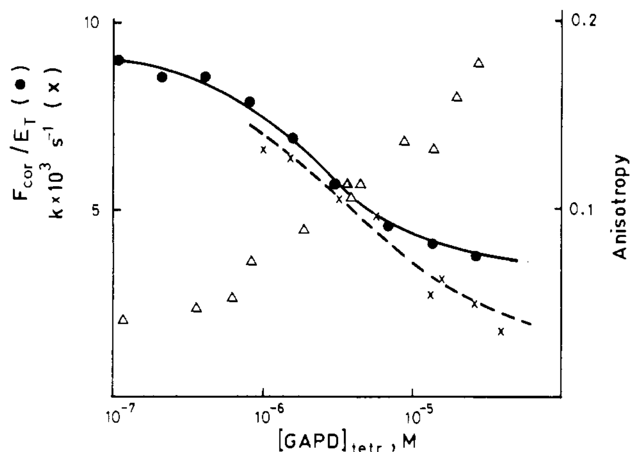

FIGURE 4: Enzyme concentration dependence of the corrected specific fluorescence (F_{cor}/E_T) intensity (●), the first-order rate constant (k) of the formation of the absorption difference of the FITC-labeled GAPD and NAD at 480 nm (×), and the fluorescence anisotropy (△) of the FITC-labeled GAPD in the presence of 1 mM NAD after 30-min incubation. The enzyme contained 1.2 mol of dye/mol of tetramer. The concentration dependences of both F_{cor}/E_T (—) and k (---) were fitted with a theoretical curve assuming a two-step dissociation model, with $K_1 = 4\ \mu M$ and $K_2 = 8\ \mu M$ for tetramer–dimer and dimer–monomer equilibria, respectively. For details of the measurements of fluorescence anisotropy, see Materials and Methods.

(A_{eq}) and the specific corrected fluorescence emission (F_{cor}/E_T) show characteristic dependence on enzyme concentration (Figure 5). A control experiment showed that in the same protein concentration range neither the specific fluorescence intensity nor the anisotropy of the FITC-labeled tetrameric aldolase changes on dilution (Ovádi et al., 1978; Batke, 1982), indicating that the spectroscopic properties of the probe itself were not affected by dilution. Therefore we assumed that the shape of both curves reflects the dissociation of tetrameric apoenzyme.

The shape and the time progress of the "dissociation-like" curves are independent of the amount of the dye bound to the enzyme (cf. Figure 5), which indicates that FITC labeling does not affect the dissociation of the enzyme. Moreover, we found that the ratio of the FITC-labeled and unlabeled enzymes did not influence the anisotropy values. These findings provide evidence that the labeled and unlabeled enzymes dissociate similarly.

As a consequence of the dissociation, both the conformation of the individual subunits in the different oligomeric forms and the rigidity of the covalently bound dye can change, which results in a dramatic decrease in the anisotropy values. Indeed, the decrease in the rotational correlation time from 24 to 0.26 ns, the values corresponding to the tetrameric and monomeric forms, respectively, is more than can be expected on the basis of the Perrin equation if the tetramer (M_r 145 000) dissociates into monomers (Table I), indicating that the monomer binds the dye less rigidly than the tetramer. This allows one to follow the dissociation on the basis of the relative fluorescence intensity vs. enzyme concentration curve.

Under the assumption that at the highest and lowest enzyme concentrations practically only tetramers and monomers exist, respectively, good estimates of the intrinsic specific fluorescence

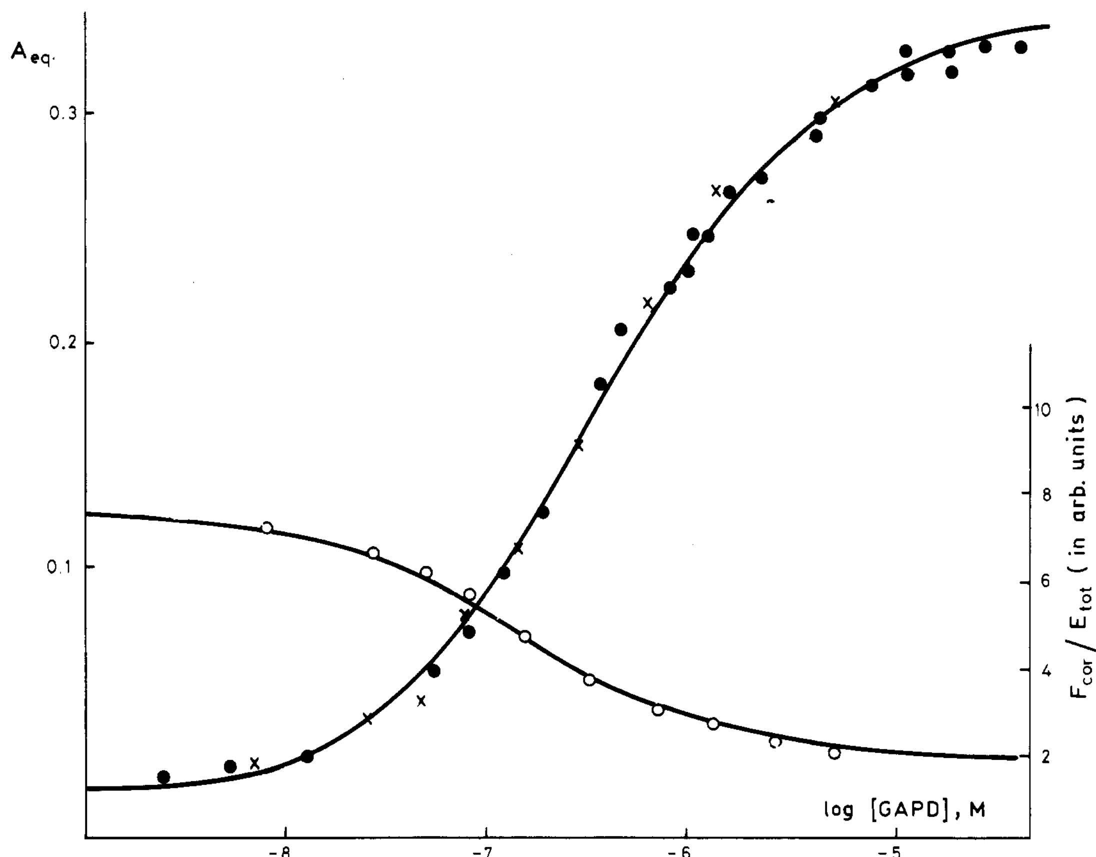

FIGURE 5: Enzyme concentration dependence of the corrected specific fluorescence intensity (○) and fluorescence anisotropy (●, ×) of FITC-labeled apo-GAPD from pig muscle. Measurements were performed at 20 °C after 1 h following dilution of the enzyme solution, when a constant value of the anisotropy had been reached. Both the concentration dependence of the corrected specific fluorescence intensity (F_{cor}/E_T) and the anisotropy (A_{eq}) were fitted with theoretical curves (solid lines) assuming the two-step dissociation of the tetramer. The quantitative data for dissociation constants are presented in Table II. For details of measurements and the theoretical calculations see the text. The symbols (●), (○) and (×) represent measurements with enzymes containing 0.3, 1.5, and 1.5 mol of FITC/tetramer, respectively.

intensities are available from the limit values for the tetramer (q_t = 2) and for the monomer (q_m = 7.4). However, an assumption has to be made for the value characteristic of the dimer (q_d = 4).

The solid line to fit the fluorescence intensity data in Figure 5 shows the theoretical curves based on the following dissociation constants: $K_1 = 1 \times 10^{-7}$ M and $K_2 = 1 \times 10^{-6}$ M, for the tetramer–dimer and dimer–monomer equilibria, respectively.

The formula proposed by Rawitch & Weber (1972) referring to one-step dissociation was extended to describe a two-step dissociation process in order to calculate the fractions of different forms (tetramer, dimer, and monomer) also from the anisotropy change as a function of enzyme concentration.

The observed anisotropy at equilibrium A_{eq} is then

$$A_{eq} = \frac{a_t q_t c_t + a_d q_d c_d + a_m q_m c_m}{q_t c_t + q_d c_d + q_m c_m} \quad (1)$$

where the subscripts t, d, and m refer to the tetrameric, dimeric, and monomeric forms, respectively. Symbol q is the relative fluorescence quantum yield, a is the intrinsic anisotropy, and c is the molar enzyme concentration in tetrameric equivalents.

The intrinsic anisotropies from the limit values (Figure 5) for tetramer and monomer were taken as 0.33 and 0.02, respectively, while for the dimer 0.18 was assumed (fitting was rather insensitive to the least value). The values of q_t, q_d, and q_m were estimated from the concentration dependence of F_{cor}/E_T presented in Figure 5.

The solid line to fit the anisotropy data in Figure 5 shows the theoretical curve calculated from eq 1 by using the following dissociation constants: $K_1 = 1 \times 10^{-7}$ M for the tetramer–dimer equilibrium and $K_2 = 5 \times 10^{-7}$ M for the dimer–monomer equilibrium. It should be noted that the anisotropy vs. enzyme concentration curve cannot be fitted by a theoretical curve assuming merely a tetramer–dimer equilibrium. However, the tetramer–monomer model can adequately accommodate the data. Minton & Wilf (1981) have drawn a similar conclusion from the decrease of enzymatic activity as a function of GAPD concentration without analyzing the two-step dissociation. Since we have found that the dissociation constant for the tetramer–dimer equilibrium is lower than that for the dimer–monomer equilibrium, the relative concentration of dimer is very low over the concentration range studied. Consequently, neither the tetramer–monomer nor the tetramer–dimer–monomer equilibrium can be experimentally supported. However, Hoagland & Teller (1969) excluded the existence of trimeric form by ultracentrifugation studies; morover, a one-step tetramer–monomer equilibrium thermodynamically is very improbable, and therefore, the postulated two-step dissociation model seems very plausible. The quantitative data of the two-step dissociation of GAPD show that the dissociation of tetramer into dimers needs more energy than that of the dimer into monomers.

Although the concentration range studied in ultracentrifugation experiments (Hoagland & Teller, 1969; Smith & Schachman, 1973; Lakatos & Závodszky, 1976) is narrow compared to that in the present fluorescence measurements, we fitted the former data with a two-step dissociation curve using dissociation constants compatible with those estimated from our fluorescence measurements (Table II).

Figure 6 shows the enzyme concentration dependence of the weight-average molecular weight (M_w) data of ultracentrifugation experiments carried out with enzymes prepared from pig (Lakatos & Závodszky, 1976) and rabbit (Smith & Schachman, 1973) muscle. A good fit to these points was found if the limit value of weight-average molecular weight at high enzyme concentration was taken to be slightly higher than that of the tetramer (i.e., 160 000) in agreement with the conclusion of Smith & Schachman (1973) that aggregates higher than the tetramer are irreversibly formed during the relatively long time (18–24 h) of ultracentrifugation run.

We have found no noticeable differences in the dissociation properties of GAPD's isolated from pig and rabbit muscle by fluorescence polarization. This finding is in good agreement with the ultracentrifugation data (cf. Figure 6).

Detection of the Dissociation of GAPD in the Presence of NAD. We have investigated how the binding of NAD influences the distribution of different oligomeric forms as the function of the enzyme concentration. Figure 4 shows how

Table II: Comparison of the Dissociation Constants Estimated from Different Measurements

method	dissociation constants (M) for: tetramer–dimer equilibrium	dimer–monomer equilibrium
fluorescence intensity	1×10^{-7}	1×10^{-6}
fluorescence anisotropy	1×10^{-7}	5×10^{-7}
ultracentrifugation [a]	5×10^{-7}	5×10^{-6}

[a] Experimental data of ultracentrifugation experiments were taken from the literature (see Figure 6) and were fitted by us as given in the text.

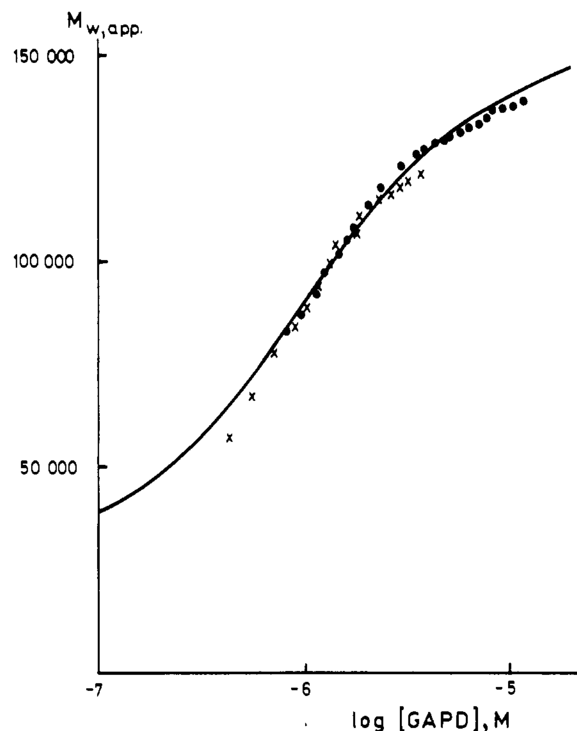

FIGURE 6: Estimation of dissociation constants by using the data of ultracentrifugation experiments taken from the literature: Lakatos & Závodszky (1976) (×) and Smith & Schachman (1973) (●). Values of the weight-average molecular weights ($M_{w,app}$) vs. the concentration of apo-GAPD were fitted by using intrinsic dissociation constants: $K_1 = 5 \times 10^{-7}$ M for tetramer–dimer and $K_2 = 5 \times 10^{-6}$ M for dimer–monomer equilibria. The molecular weights of tetramer, dimer, and monomer were taken to be 145 000, 72 500, and 36 250, respectively. However, for the limit of the theoretical curve at high concentrations, 160 000 was taken as the molecular weight.

the specific fluorescence intensities (F_{cor}/E_T) increase with decrease in enzyme concentration in the presence of 1 mM NAD, which is sufficient to saturate the enzyme at all concentrations studied. The corrected specific fluorescence intensity as a measure of the relative quantum efficiency shows a dependence on enzyme concentration. Under the assumption that this change represents the dissociation of the tetramers into dimers and monomer, similarly to apo-GAPD, the experimental points can be fitted to a two-step dissociation model. The estimated dissociation constants are $K_1 = 4 \times 10^{-6}$ M and $K_2 = 8 \times 10^{-6}$ M for the tetramer–dimer and dimer–monomer equilibria, respectively. With the same dissociation constants the change of k in function of enzyme concentration could also be fitted in the measured concentration range (Figure 4).

The fluorescence anisotropy of FITC–GAPD was also measured over a wide range of protein concentration in the presence of 1 mM NAD. Figure 4 shows that the anisotropy of the labeled enzyme, measured after it had reached a constant value, is decreased by dilution of the labeled protein. Therefore we assume that the change of anisotropy as a function of enzyme concentration probably also reflects the dissociation of GAPD even in the presence of excess NAD (Ovádi et al., 1979). However, the estimation of dissociation constants in the presence of excess NAD from the anisotropy vs. enzyme concentration curve is rather uncertain since the measurement of the limit value of anisotropy was unfeasible experimentally at high enzyme concentrations; hence, the number of unknown parameters to fit the anisotropy vs. enzyme concentration curve by eq 1 is also increased as compared to the number of unknowns in the experiments with apoenzyme.

We have checked the effect of bound FITC on the dissociation–association properties of the enzyme in the presence of 1 mM NAD and found that the binding of FITC did not influence it.

GAPD and FITC-labeled GAPD at a concentration of 1 μM were gel filtered on a Sephadex G-200 column equilibrated with 50 mM Tris-HCl buffer containing 1 mM EDTA and 1 mM NAD, pH 8.5 (Figure 7). In both cases the elution profiles were symmetrical, and the position of the peaks was practically identical and fell between those of tetramer and dimer, which indicates a reversible, rapid equilibrium between these forms. The recovered activities were about 90%. This experiment indicates qualitatively the dissociation of tetrameric GAPD in the presence of 1 mM NAD, but such an experiment is inadequate to determine dissociation constants (Valdes & Acker, 1979).

In conclusion, the experience that the covalently bound FITC does not alter the functional and dissociation–association properties of GAPD validates the use of this dye in the study of the oligomerization of GAPD. The values of dissociation constants for tetramer–dimer and dimer–monomer equilibria are in the micromolar range as calculated from the enzyme concentration dependence of the specific fluorescence intensities and k.

The dissociation constant for the tetramer–dimer equilibrium estimated by us is compatible with that determined by Hoagland & Teller (1969) by ultracentrifugation. However, Lakatos & Závodszky (1976) found that in the range of 10–0.1 μM GAPD the enzyme can exist practically only in the tetrameric form in the presence of 1 mM NAD.

We think the controversy is not due to the different sources of enzyme preparations since we have repeated all typical experiments with rabbit muscle enzyme and got the same qualitative picture.

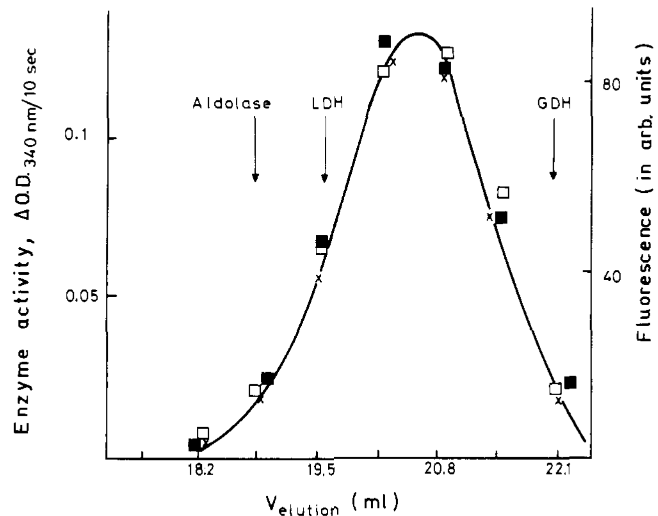

FIGURE 7: Elution profiles of GAPD (■) and GAPD–FITC (□ and ×) in the presence of 1 mM NAD. Gel chromatographic runs were made on a Sephadex G-200 column (0.6 × 96 cm) at 4 °C equilibrated with 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 1 mM NAD. 1.2-mL 1 μM GAPD and FITC-labeled GAPD (1.9 mol of dye/mol of tetramer) solutions were loaded. Symbol (×) shows the fluorescence intensity of FITC-labeled samples. The arrows marked the elution maxima of aldolase (M_r 160 000), lactate dehydrogenase (LDH) (M_r 140 000), and glycerolphosphate dehydrogenase (GDH) (M_r 78 000). All experiments were carried out separately on the same column. For other details see the text and Materials and Methods.

The labeling of dehydrogenase with fluorescent dye allowed us to follow the local conformational changes caused by successive NAD binding.

We suggest that the NAD-induced local conformational changes manifest themselves in the fluorescent dye's environment, located outside the active center of GAPD, and are transmitted through the contact surfaces of subunits without altering remarkably the strength of subunit interactions, that is, the dissociation of the tetrameric enzyme. Further, we have succeeded in detecting an additional NAD-induced conformational alteration by elevation of NAD concentration beyond that needed for stoichiometric binding. This observation implies the existence of a second NAD binding site(s) per subunit. Therefore GAPD may have not only a "relaxed" and a "tight state" owing to apo- and holoenzyme, respectively, but also a third, stable state, the "compact" one, having additional bound NAD molecules outside the active center. Finally we can speculate about the possible physiological role of the dissociation of GAPD. The relevance of dissociation in vivo from observation in vitro ought to be treated with particular caution. The level of GAPD in living cells can be considered constant; therefore, changes of enzyme concentration are to be neglected. However, if we take into account that the substrates can induce dissociation (Kálmán et al., 1980) and the catalytic properties of the dissociated forms differ from those of the tetramer (Ovádi et al., 1979; Jancsik et al., 1979; Minton & Wilf, 1981), then the regulatory role is not unreasonable. In addition, we must consider that this enzyme (GAPD) can associate with other glycolytic enzymes (Ovádi & Keleti, 1978; Ovádi et al., 1978; Patthy & Vas, 1978; Pontremoli et al., 1979; Grazi & Trombetta, 1980; Földi et al., 1973) and with some elements of the contractile apparatus or membranes (Arnold & Pette, 1968; Clarke & Masters, 1974; Cseke et al., 1978; Solti & Friedrich, 1976). These phenomena can create a situation in the cell when the concentration of the unbound GAPD dramatically falls, resulting in its spontaneous dissociation.

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