Accessibility of Adenine Binding Sites in Dehydrogenases to Small Molecules Studied by Fluorescence Quenching†

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ABSTRACT: Quenching of the fluorescence of ethenoadenine derivatives by iodide ions and by methionine was studied in solution and when the nucleotides were bound to several dehydrogenases. The fluorescence of ϵADPR in neutral aqueous solution is dynamically quenched by both quenching agents. The quenching of free ϵNAD$^+$ by methionine was found to be predominantly static and was satisfactorily described to result from complex formation between quencher and dinucleotide. The rate constant for quenching by iodide of ϵNAD$^+$ in the ternary complex with LADH and pyrazole is comparable to that of free ϵADPR or ϵADP. It is concluded that the bound ϵ-adenine ring is partially exposed to the solvent. The opening, to the solvent, of the adenine binding site is not large enough to allow free methionine diffusion since the rate constant for quenching of bound coenzyme by this quenching agent is relatively small. The difference between the rate constants for quenching of free and enzyme bound nucleotide was used to evaluate the binding constants of ϵADPR to GPDH, ϵNAD$^+$ to LDH, and oxalate to the LDH:ϵNAD$^+$ complex. This technique may prove to be particularly useful when the binding of a fluorescent ligand to a protein is not accompanied by significant changes in its fluorescence.

The fluorescent coenzyme analogue ϵNAD$^+$ [1] has been used by several workers to probe the adenine binding sites of NAD$^+$-linked dehydrogenases as well as the conformation of the bound coenzyme analogue (Lee & Everse, 1973; Schlessinger & Levitzki, 1974; Schlessinger et al., 1975; Luisi et al., 1975; Gafni, 1977). The pronounced enhancement of fluorescence of ϵNAD$^+$ upon binding was used to follow the binding mechanism and to determine the dissociation constants. Free ϵNAD$^+$, in aqueous solution, was found to be in an equilibrium between folded and open conformations (Lee & Everse, 1973; Gruber & Leonard, 1975; Gafni, 1977). In the folded conformation partial quenching of the fluorescence is caused by stacking of the ethenoadenine and nicotinamide rings. The similar values observed for the enhancement of ϵNAD$^+$ fluorescence upon binding to several dehydrogenases led Luisi et al. (1975) to conclude that the coenzyme is bound in the unfolded conformation.

Collisional quenching of fluorescent groups bound to a protein molecule may yield direct information on the rate of penetration of the quenching agent to the vicinity of the fluorophore (Lehrer, 1967, 1971). Using quenching agents which differ in size or charge, one can study the effects of these properties on the rate of diffusion of quencher into the protein. Shaklai et al. (1978) who studied iodide quenching of tryptophan fluorescence in hemocyanin concluded that iodide effectively quenched only the fluorescence of tryptophan residues near the exterior of the protein molecule. The fluorescence of tryptophan residues located inside the protein molecule was not affected. Harvey & Cheung (1976) found no quenching of ϵATP fluorescence by either iodide or methionine when the nucleotide was bound to G-actin. The nucleotide base was thus concluded to be inaccessible to the quenching agents.

In the present study we describe the use of small molecules as quenchers for ϵNAD and ϵADPR fluorescence both in solution and when bound to several dehydrogenases. In addition to the information gained about the accessibility of the binding sites to these quenching reagents, the data are used to determine the binding constants for the enzyme–ethenoadenine derivative complex. This technique is very useful in cases where the changes in fluorescence upon binding are small.

Materials and Methods

Horse liver alcohol dehydrogenase and beef heart lactate dehydrogenase were obtained from Boehringer Mannheim Co., as crystalline suspensions. The suspensions were centrifuged and the precipitated proteins dissolved in 0.05 M phosphate buffer, pH 7.3, and dialyzed against several changes of the same buffer for 48 h. Enzyme concentrations were determined from the optical density at 280 nm (Sund & Theorell, 1963; Velick, 1958). Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was kindly donated by Dr. A. Levitzki and Y. Henis (the Hebrew University, Jerusalem). Apo-GPDH was prepared by treating the holoenzyme (in 0.05M Hepes buffer, pH 7.4, containing 10 mM EDTA) with HCl-treated Norit A as described by Schlessinger & Levitzki (1974). The apoenzyme had an A_{280}/A_{260} ratio of 1.80–1.85, indicating that it was substantially free of NAD$^+$. NADase (from *N. crassa*) was obtained from Sigma as a solid extract and used without further purification.

ϵNAD$^+$ was purchased from Sigma and its purity determined by thin-layer chromatography (Eastman cellulose sheets) according to Secrist et al. (1972). The nucleotide concentration was determined spectrophotometrically using an extinction coefficient at 265 nm of 1.0×10^4 M^{-1} cm^{-1} (Luisi et al., 1975). ϵADP was obtained from Sigma and used without further treatment. ϵADPR was prepared from ϵNAD$^+$ by cleavage with NADase. The contents of one vial of NADase (~0.7 mg) was dissolved in 2 mL of 2×10^{-4} M ϵNAD$^+$ solution in 50 mM Hepes buffer (pH 7.4) containing 10 mM EDTA. The mixture was kept for 1 h at room temperature (~23 °C) and the reaction followed using the marked enhancement of fluorescence intensity upon cleavage. Complete cleavage was reached in about 30 min.

Ternary complexes of ϵNAD$^+$ with LADH and pyrazole were prepared by adding the required volume of 2×10^{-4} M ϵNAD$^+$ solution in 0.05 M phosphate buffer (pH 7.3) con-

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[1] Abbreviations used: ϵNAD$^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide; ϵADPR, 1,N^6-ethenoadenosine diphosphoribose; ϵADP, 1,N^6-ethenoadenosine diphosphate; ϵATP, 1,N^6-ethenoadenosine triphosphate; LADH, horse liver alcohol dehydrogenase; LDH, beef heart lactate dehydrogenase; GPDH, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

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Table I: Fluorescence Decay Times and Rate Constants for Fluorescence Quenching of Ethenoadenine Derivatives[a]

ethenoadenine derivative	quencher	quencher concn (mM)	F_0/F	decay time (ns)	τ_a/τ	quenching rate constant (M^{-1} s^{-1})
εADP in neutral aq soln	KI	0	1.0	24.3	1.0	4.1×10^9
		40	5.03	4.7	5.2	
εADPR in neutral aq soln	KI	0	1.0	22.4[b]		3.9×10^9
		40	4.5			
	Met	0	1.0	22.4[b]		7.4×10^8
		75	2.25	9.7[b]	2.31	
GPDH:εADPR	Met	0	1.0	22.6[b]		6.0×10^7
		52	1.07			
LDH:εNAD$^+$:oxalate (50 mM)	Met	0	1.0	25.6		2.7×10^7
		100	1.07			
LADH:εNAD$^+$:pyrazole	KI	0	1.0	28.6	1.0	2.5×10^9
		40	3.8	8.6	3.3	
	Met	0	1.0			5.9×10^7
		100	1.17			

[a] The rate constants were calculated by use of eq 1. τ_a/τ is the ratio between the decay times in absence and presence of quencher in the concentration indicated. The good correspondence between this value and the value of F_0/F confirms that the quenching is dynamic. [b] A second decay component (of about 1 ns) with a low amplitude was present in this case and apparently originated in fluorescent materials in the NADase extract used in the εADPR preparation.

taining 1.7 mM pyrazole to a 1.2×10^{-5} M solution of LADH in the same buffer–pyrazole solution. The complexes were prepared with a binding sites to εNAD$^+$ ratio of 2.

Ternary complexes of LDH:εNAD$^+$:oxalate were prepared in a similar way, the final concentrations of enzyme and εNAD$^+$ being 1.0×10^{-5} and 1.2×10^{-5} M, respectively (i.e., binding sites to εNAD$^+$ ratio of 3.3). Three oxalate concentrations were used in preparing these complexes and were 50, 6.7, and 0.27 mM. GPDH:εADPR complexes were prepared by adding the required volume of a 2×10^{-4} M εADPR solution in 50 mM Hepes buffer (pH 7.4) containing 10 mM EDTA to a 7.4×10^{-5} M GPDH solution in the same buffer. The molar ratio of εADPR to GPDH was 1:1.

Fluorescence spectra were measured with a Perkin-Elmer MPF-3 spectrophotofluorometer using a half-band width of 5 nm in excitation and emission. Two fluorescence quenching agents were used, iodide ions (from a stock solution of 2 M potassium iodide) and DL-methionine (added from a 200 mM stock solution). The fluorescence quenching was studied by monitoring the intensity of fluorescence of 400 nm, excited by 315-nm light, with changing quencher concentration (both iodide and methionine caused no shift or change of shape of the fluorescence, and hence monitoring the intensity at one wavelength was sufficient). Fluorescence decay measurements were done using an instrument built in this laboratory and described elsewhere (Hazan et al., 1974). The excitation wavelength was 315 nm and the fluorescence was observed through a Schott KV394 cut-off filter transmitting light of wavelengths above ~400 nm. The decay curves were analyzed for one or two exponential components using the method of nonlinear least squares (Grinvald & Steinberg, 1974). All the experiments were done at room temperature (~23 °C).

Results

The fluorescence of εNAD$^+$ is markedly enhanced upon cleavage by NADase to form εADPR, as shown in Figure 1. The fluorescence maximum is not affected by the cleavage. This fluorescence enhancement is due to the elimination of the folded conformation where the interaction of the nicotinamide and ethenoadenine rings reduces the fluorescence of the latter. A similar enhancement of the fluorescence intensity has been observed for εNAD$^+$ cleaved by phosphodiesterase to form εADP or when bound to several dehydrogenases (Luisi et al., 1975), indicating a fully unfolded conformation of the bound coenzyme analogue.

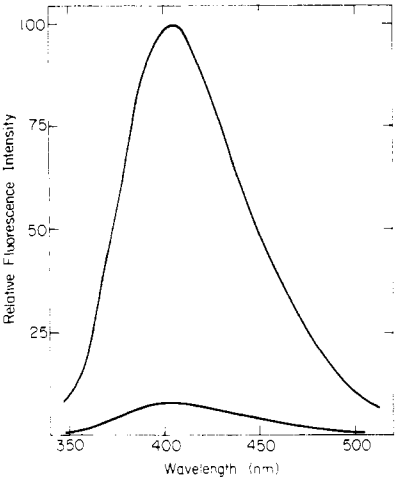

FIGURE 1: Emission spectra of εNAD$^+$ (lower line) and of the same sample after cleavage by NADase (upper line). The sample was dissolved in 50 mM Hepes buffer (pH 7.4) containing 10 mM EDTA. The excitation wavelength was 315 nm.

Figure 2 presents fluorescence quenching data for εADPR and εADP in aqueous solution and of εNAD$^+$ in the ternary complex with LADH and pyrazole. The quenching both by iodide ions and by methionine clearly follows a Stern–Volmer relationship indicative of dynamic (collisional) quenching. This mechanism for quenching is supported by results of fluorescence decay measurements which show the expected shortening in the decay constants upon addition of quencher (see Table I).

From the Stern–Volmer plots presented in Figure 2 and the decay constants given in Table I, the rate constants for fluorescence quenching of the ethenoadenine ring in the systems studied may be calculated using the Stern–Volmer equation

$$F_0/F = 1 + k_q\tau_a[Q] \quad (1)$$

where τ_a is the fluorescence decay constant in the absence of quencher, [Q] is the concentration of quencher, and k_q is the quenching rate constant. The values of k_q for the systems shown in Figure 1 are summarized in Table I.

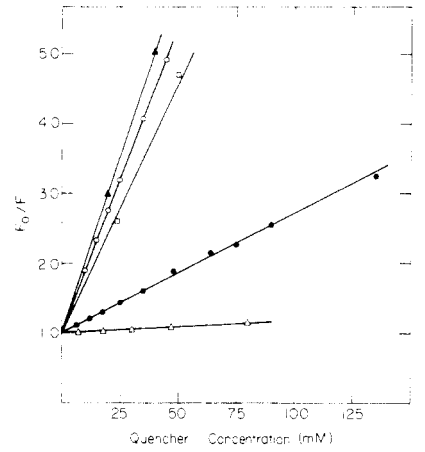

FIGURE 2: Stern–Volmer plots of some ethenoadenine derivatives using iodide and methionine as fluorescence quenching agents. All the experiments were done in neutral aqueous buffer solutions as described in the text. (▲) ϵADP fluorescence as quenched by iodide; (○) ϵADPR fluorescence quenched by iodide; (□) the ternary complex LADH:ϵNAD$^+$:pyrazole; quenching agent, iodide; (●) ϵADPR fluorescence quenching by methionine; (△) fluorescence quenching of the LADH:ϵNAD$^+$:pyrazole ternary complex by methionine.

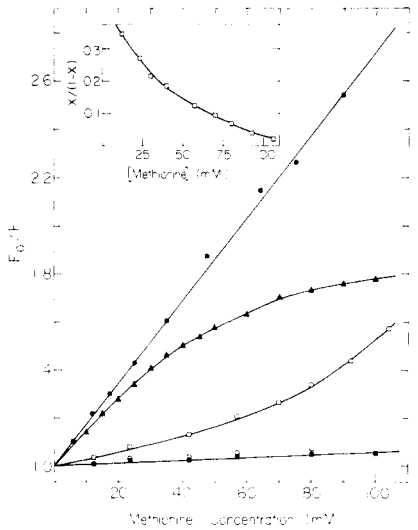

FIGURE 3: Fluorescence quenching by methionine of ϵADPR (●), ϵNAD$^+$ (▲), and of the ternary complex LDH:ϵNAD$^+$:oxalate using the following (initial) oxalate concentrations: 50 mM (■), 6.7 mM (○), 0.27 mM (□). More details on the experimental conditions are given in the text. Insert: the effect of methionine additions to the LDH:ϵNAD$^+$:oxalate (0.27 mM) ternary complex on the ratio between the concentrations of bound and free ϵNAD$^+$.

Figure 3 presents the fluorescence quenching by methionine of ϵNAD$^+$ in aqueous solution and in the LDH:ϵNAD$^+$:oxalate complex as compared with ϵADPR quenching by the same reagent. The fluorescence decay constant of free ϵNAD$^+$ in aqueous solution is about ten times smaller than that of ϵADPR. The values of F_0/F expected from eq 1 are therefore much smaller than the observed ones indicating that the quenching is not dynamic. Also the quenching of free ϵNAD$^+$ markedly deviates from the linear dependence on quencher concentration of the Stern–Volmer equation. This deviation is also indicative of a static quenching mechanism, i.e., formation of a (ground state) complex between ϵNAD$^+$ and methionine. This may be described as

$$\epsilon\text{NAD}^+ + \text{M} \rightleftarrows \text{P} \qquad K_p = \frac{[\text{P}]}{[\epsilon\text{NAD}^+][\text{M}]} \qquad (2)$$

M being methionine and P its complex with ϵNAD$^+$. Since

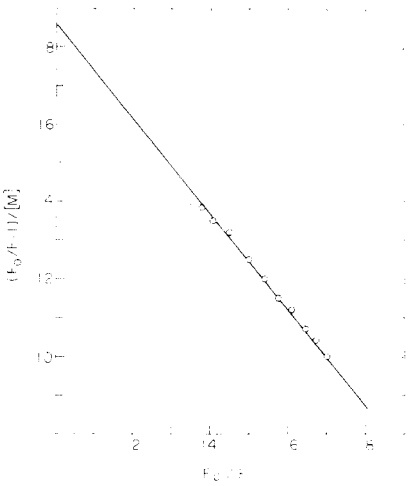

FIGURE 4: The fluorescence quenching data of ϵNAD$^+$ (F_0/F, from Figure 3) plotted according to eq 3. [M], methionine concentration (mol/L). The binding constant, K_p, of the ϵNAD$^+$–methionine complex, and its relative fluorescence intensity, compared with that of ϵNAD$^+$, were evaluated from the slope and intercept of the straight line to be 31 M^{-1} and 0.41, respectively.

methionine was added in large excess over coenzyme, the concentration of the unbound species, [M], was practically equal to the total concentration of methionine added. Denoting by F_ϵ the fluorescence intensity, under the experimental conditions used, of 1 mol/L ϵNAD$^+$, and similarly by F_p that of 1 mol/L ϵNAD$^+$–methionine complex, one obtains

$$F = F_\epsilon[\epsilon\text{NAD}^+] + F_p[\text{P}]$$

and in the absence of methionine

$$F_0 = F_\epsilon[\epsilon\text{NAD}^+]_0$$

$[\epsilon\text{NAD}^+]_0$ is the initial concentration of ϵNAD$^+$ in solution.

Since $[\epsilon\text{NAD}^+]_0 = [\epsilon\text{NAD}^+] + [\text{P}]$, it follows from the equation given above that

$$\frac{1}{[\text{M}]}[(F_0/F) - 1] = K_p\left(1 - \frac{F_p}{F_\epsilon} \times \frac{F_0}{F}\right) \qquad (3)$$

Thus a plot of $(1/[\text{M}])[(F_0/F) - 1)$ as a function of F_0/F should result in a straight line with a slope of $-K_pF_p/F_\epsilon$ and an intercept of $K_p(1 - F_p/F_\epsilon)$. This is plotted in Figure 4 and the values of K_p and F_p/F_ϵ calculated from the slope and intercept are 31 M^{-1} and 0.41, respectively. The fluorescence of the ϵNAD$^+$–methionine complex is thus reduced about 2.5-fold compared with that of the free dinucleotide.

The fluorescence quenching experiments of LDH:ϵNAD$^+$:oxalate ternary complexes by methionine presented in Figure 3 were done using three different oxalate concentrations. Since the added methionine solution contained no oxalate, the concentration of the latter in the samples under study decreased, due to dilution with the additions of quencher solution. The concentrations quoted in Figure 3 are those of the initial solutions before methionine addition. The changes in oxalate concentration were used to calculate its binding constant to the LDH:ϵNAD$^+$ complex as will be described in the following.

The formation of the ternary complex between LDH, ϵNAD$^+$, and oxalate may be described as

$$\text{LDH} + \epsilon\text{NAD}^+ \overset{K_b}{\rightleftharpoons} \text{LDH}{:}\epsilon\text{NAD}^+ + \text{oxalate} \overset{K_{ox}}{\rightleftharpoons} \text{LDH}{:}\epsilon\text{NAD}^+{:}\text{oxalate}$$

Denoting by K_b the binding constant of ϵNAD$^+$ to LDH and by K_{ox} the binding constant of oxalate to the binary LDH:

ϵNAD$^+$ complex to form the ternary complex, one obtains

$$K_{app} = \frac{[LDH{:}\epsilon NAD^+] + [LDH{:}\epsilon NAD^+{:}Ox]}{[LDH][\epsilon NAD^+]} = K_b(1 + K_{ox}[ox]) \quad (4)$$

K_{app} is the apparent binding constant of ϵNAD$^+$ to the enzyme in presence of oxalate and is seen to increase with increasing oxalate concentration. The quenching curves of Figure 3 clearly show that while at the two higher oxalate concentrations used the fluorescence intensity is only slightly affected by methionine, at the lower oxalate concentration the quenching agent has a marked effect on the fluorescence. This results from the different extents to which the fluorescence of free and LDH bound ϵNAD$^+$ is quenched by a given methionine concentration. From the very small difference between the quenching curves in presence of 6.7 and 50 mM oxalate, we conclude that at these concentrations K_{app} is large enough so that practically all of the ϵNAD$^+$ is bound, and hence only one fluorescent species is present. The small quenching by methionine observed in presence of 50 mM oxalate was used to evaluate the rate constant, k_q, and a value of 2.7×10^7 M^{-1} s^{-1} was obtained.

The larger quenching effect observed in presence of 0.27 mM oxalate indicates that the coenzyme analogue is only partially bound to LDH. From this quenching curve the ratio between free and LDH bound ϵNAD$^+$ may be calculated in the following way. The fluorescence of coenzyme analogue bound to LDH in the binary as well as in the ternary complex with oxalate was found to be 12.5 times larger than that of free ϵNAD$^+$. Denoting the relative concentration of complexed ϵNAD$^+$ by x, one may write for the fluorescence intensity in absence of methionine

$$F_0 = K[(1 - x) + 12.5x] = K(1 + 11.5x)$$

K is a constant depending on the instrumental setting. In presence of M mol/L methionine

$$F_M = K\left[\frac{1 - x}{(F_0/F)_M{}^F} + \frac{12.5x}{(F_0/F)_M{}^B}\right]$$

where $(F_0/F)_M{}^F$ is the value of fluorescence quenching for free ϵNAD$^+$ in presence of M mol/L methionine and $(F_0/F)_M{}^B$ is the corresponding value for the bound coenzyme (i.e., from the data in presence of 50 mM oxalate). From the values of $(F_0/F)_M$, $(F_0/F)_M{}^F$, $(F_0/F)_M{}^B$, shown in Figure 3, the ratio $x/(1 - x)$ was calculated for various methionine concentrations and is presented in the insert to this figure. The relative concentration of bound coenzyme is seen to drop sharply with increasing methionine concentration. In calculating the value of K_{app} from the plot of $x/(1 - x)$ values vs. methionine concentration, it should be borne in mind that the term $1 - x$ includes both free and methionine bound ϵNAD$^+$ (P), while in eq 4 only free ϵNAD$^+$ is involved. The relative concentration of the latter may be derived using eq 2 and is

$$\frac{[\epsilon NAD^+]}{[\epsilon NAD^+] + [P]} = \frac{1}{K_p[M] + 1}$$

For K_{app} one thus finally obtains

$$K_{app} = \frac{x}{(1 - x)[LDH]}(1 + K_p[M]) \quad (5)$$

K_{app} is dependent on oxalate concentration (see eq 4). The latter was changing with the additions of methionine solution due to dilution (the methionine solution contained no oxalate). A plot of K_{app} vs. oxalate concentrations is shown in Figure 5. The values of K_b and K_{ox} calculated from the intercept and slope of this plot by use of eq 4 were found to be 2.4×10^4 and 5.6×10^3 M^{-1}, respectively. Using these values it is found that in presence of 50 mM oxalate more than 98% of the ϵNAD$^+$ molecules are bound to LDH. Since the fluorescence of ϵNAD$^+$ bound to the enzyme is enhanced 12.5 times compared with that of the free coenzyme analogue, only about 0.2% of the fluorescence originates in free ϵNAD$^+$ molecules. This figure fully agrees with the previously stated conclusion that at this oxalate concentration only fluorescence of bound ϵNAD$^+$ is observed.

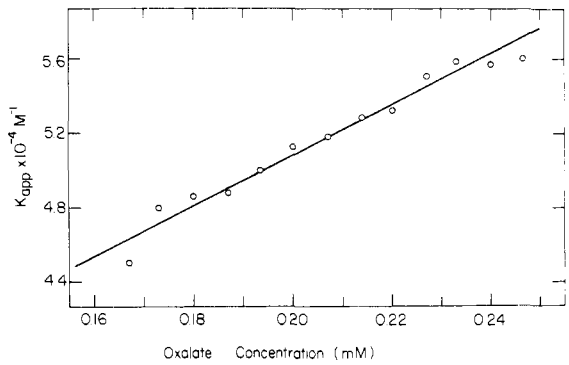

FIGURE 5: The apparent binding constant, K_{app}, of ϵNAD$^+$ to LDH in presence of oxalate as a function of oxalate concentration. The values of K_{app} were calculated by means of eq 5. From the intercept and slope of the straight line the binding constants of ϵNAD$^+$ to LDH (K_b) and of oxalate to the LDH:ϵNAD$^+$ binary complex K_{ox} were evaluated by means of eq 4 to be 2.4×10^4 M^{-1} and 5.6×10^3 M^{-1}, respectively.

Table II: Fluorescence Quenching Results of ϵADPR-GPDH Mixtures by Methionine[a]

GPDH concn (mol/L)	ϵADPR concn (mol/L)	Met concn (mM)	F_0/F
4.6×10^{-5}	4.6×10^{-5}	0	1
3.6×10^{-5}	3.6×10^{-5}	52	1.30
1.07×10^{-5}	1.05×10^{-5}	28	1.32
0.92×10^{-5}	0.91×10^{-5}	52	1.56

[a] More details are given in the text.

When ϵADPR was added to GPDH, only very small changes in the nucleotide fluorescence could be detected. Addition of methionine, however, resulted in smaller quenching than expected for free ϵADPR, indicating that ϵADPR was at least partially bound to the enzyme. The quenching data are shown in Table II. The values of F_0/F given in the table should not be regarded as corresponding to the (pure) GPDH:ϵADPR complex since there is no indication that the nucleotide was fully bound. Allowing for this possibility of partial binding the observed fluorescence quenching of the samples is given by

$$F_0/F = \frac{1}{\frac{1 - x}{(F_0/F)_{\epsilon ADPR}} + \frac{x}{(F_0/F)_{E:\epsilon ADPR}}} \quad (6)$$

where $(F_0/F)_{\epsilon ADPR}$ is the (known) value of fluorescence quenching of free ϵADPR and $(F_0/F)_{E:\epsilon ADPR}$ is the (unknown) ratio for the GPDH:ϵADPR complex. These two ratios depend only on methionine concentration. x is the fraction of ϵADPR bound to the enzyme and is dependent on enzyme concentration

$$K_b = \frac{x}{[1 - x]}[GPDH] \rightarrow x = \frac{K_b[GPDH]}{1 + K_b[GPDH]}$$

By substituting this expression for x into eq 6 and using two

values of F_0/F from Table II which were obtained for different enzyme concentrations but the same methionine concentration (of 52 mM) two equations are obtained from which the following values were found: $K_b = 4.2 \times 10^4$ M^{-1}; $(F_0/F)_{E:\epsilon ADPR}^{52mM} = 1.07$.

Assuming a dynamic quenching mechanism for the GPDH bound nucleotide, one obtains from the last figure and the fluorescence decay time of bound ϵADPR a value for the quenching rate constant, $k_q = 6.0 \times 10^7$ M^{-1} s^{-1}.

Discussion

The fluorescence of ϵADPR (and ϵADP) in aqueous solution is dynamically quenched by both iodide ions and by methionine. The quenching reactions in both cases are diffusion controlled. The rate constant for iodide quenching of ϵNAD$^+$ in the ternary complex with LADH and pyrazole (see Table I) was found to be comparable to the rate constant for quenching of the free nucleotide in aqueous solution.

Such an efficient quenching of the fluorescence of a fluorophore bound to a protein is uncommon. Luk (1971) found that the fluorescence of pyrene in the apomyoglobin: pyrene complex was not quenched by molecular oxygen and concluded that the bound dye was well shielded from oxygen. Shaklai et al. (1978) found that iodide effectively quenched tryptophan residues on the exterior of hemocyanin, while the fluorescence of tryptophan residues "buried" in the hydrophobic interior of the protein was unaffected. Recently Harvey & Cheung (1976) reported no iodide quenching of ϵATP fluorescence when the nucleotide was bound to G actin. They concluded that the adenine binding site was inaccessible to the quenching agent. Our results suggest that the adenine binding sites in LADH are accessible to iodide ions. It is well documented that the adenine moiety of NAD is bound to LADH in a hydrophobic pocket and that there are no specific interactions which are unique for adenine but rather a general hydrophobic binding of aromatic molecules (Bränden et al., 1975). The efficient quenching of the ethenoadenine fluorescence by iodide cannot, therefore, be due to the existence of a polar adenine binding site that might have facilitated iodide diffusion but rather to the fact that the (hydrophobic) site is very close to the surface of the enzyme and not shielded from the exterior. The adenine ring must be at least partially in contact with the solvent, thus allowing iodide to collide with it and cause the observed fluorescence quenching. This conclusion is in full agreement with affinity chromatography studies which revealed that the amino group of adenine bound to LADH points out from the binding site into the solution (Mosbach et al., 1972; Lindberg et al., 1973).

Quenching by Methionine. In solution the fluorescence of ϵADPR is quenched dynamically and with a diffusion-controlled rate constant. The fluorescence quenching of ϵNAD$^+$, on the other hand, is quantitatively described by a static mechanism (i.e., due to a partially quenched ground state complex between methionine and the dinucleotide). The fact that dynamic quenching was not observed in this case is most probably due to the much shorter fluorescence decay time (more than tenfold) of ϵNAD$^+$ compared with ϵADPR. This drastically reduces the dynamic quenching effect (see eq 1). Also the formation of the ϵNAD$^+$–methionine complex largely reduces the relative concentration of free coenzyme resulting in further decrease in the dynamic fluorescence quenching. The apparent absence of dynamic quenching of ϵNAD$^+$ fluorescence by methionine may therefore be due to the smallness of the effect rather than to a small k_q.

Since methionine forms a complex with ϵNAD$^+$ but not with ϵADPR, it is clear that the nicotinamide ring plays a major role in the complex formation. The quenching of fluorescence of this complex indicates that the adenine ring is also involved in the binding. The ϵNAD$^+$ molecule is known to exist in aqueous solution predominantly in a folded conformation in which the ϵ-adenine and nicotinamide rings are stacked (Lee & Everse, 1973; Gruber & Leonard, 1975; Gafni, 1977). This stacking results in quenching of the ϵ-adenine fluorescence. Binding of methionine may cause increased interactions between the two bases of ϵNAD$^+$ leading to the observed larger quenching. Alternatively complex formation may involve intercalation of methionine between the two bases, in which case the fluorescence quenching is caused directly by the intercalated methionine.

It is well known that ϵNAD$^+$ fully assumes the unfolded conformation when bound to both LDH and LADH (Luisi et al., 1975; Gafni, 1977). There is, therefore no direct interaction between the adenine and nicotinamide rings of the bound coenzyme analogue. The rate constants for fluorescence quenching of ϵNAD$^+$ bound to these enzymes may therefore be compared to that of ϵADPR (or ϵADP) quenching. Such a comparison shows the rate constants of the bound ϵNAD$^+$ to be considerably smaller than that of free ϵADPR. Similar results are obtained when the fluorescence quenching of ϵADPR bound to GPDH is compared with that of the free nucleotide.

As was discussed above the iodide quenching experiments reveal that the adenine binding sites in LADH are partially open to the solvent. The relatively small rate constant for quenching by methionine indicates that this opening while large enough to allow fast diffusion of iodide considerably slows the bulky methionine. In fact the rate constants for quenching by methionine found for all three enzymes studied are within the range of binding rate constants (k_{on}) for substrates of this size to enzymes (Hammes & Schimmel, 1970).

It is interesting to compare the values of k_q obtained for the three dehydrogenases. Two of these, i.e., for LADH and GPDH, are similar and more than twice that of LDH. Schlessinger et al. (1975) who studied circular polarization of the luminescence (CPL) of ϵNAD$^+$ bound to several dehydrogenases found no differences between the spectra of the coenzyme analogue when bound to LADH and LDH whereas the spectrum of ϵNAD$^+$ bound to GPDH differed markedly. Their conclusion was that the adenine binding sites in the first two enzymes were very similar, if not identical, while those of GPDH were structurally different. The rate constants found in the present study show that judging by the rates of quencher diffusion into the adenine binding sites the enzymes are grouped differently. Obviously the location of a binding site inside the enzyme is of great importance to the quenching efficiency. As a result the accessibility of the very similar sites in LDH and LADH (as concluded from the CPL spectra) to methionine is quite different.

Fluorescence quenching by methionine was used in the present study also to evaluate some of the binding constants involved. The binding constant of ϵNAD$^+$ to LDH found, 2.4×10^4 M^{-1}, is similar to the one reported by Lee & Everse (1973) for the binding of ϵNAD$^+$ to chicken M_4LDH (these authors found a value of 4×10^4 M^{-1}). The binding constant of NAD$^+$ to LDH is 3×10^3 M^{-1} (Schwert & Winer, 1963); hence the ethenoadenine derivative has about tenfold higher affinity toward LDH.

The binding of ϵADPR to GPDH does not give rise to any significant change in the intensity of the nucleotide fluorescence. This is in sharp contrast to the marked enhancement of ϵNAD$^+$ emission upon binding to GPDH or to other de-

hydrogenases as found by Luisi et al. (1975). These authors found, however, that changes in the solvent microenvironment of the ϵ-adenine group did not play an important role in the fluorescence enhancement. The latter was concluded to result from opening of the stacked coenzyme conformation upon binding. A similar enhancement was found to accompany cleavage of the dinucleotide, a process which completely removes the nicotinamide. Since ϵADPR lacks the nicotinamide ring, no unstacking occurs upon its binding to GPDH and no fluorescence enhancement is observed. The marked difference between the rates of fluorescence quenching of free and bound ϵADPR thus presents a sensitive and useful method to follow the binding. This technique may prove to be useful in other cases where ligand binding is not accompanied by significant changes in the fluorescence.

The binding constant of ϵADPR to GPDH found by us (4.2×10^4 M^{-1}) is considerably smaller than the one reported by Schlessinger & Levitzki (1974) for the binding of the first ϵNAD^+ molecule to GPDH (i.e., $K_1 \gg 10^6$ M^{-1}). A similar relation was found between the binding constant of the native coenzyme (i.e., NAD) and ADPR to GPDH. The former was estimated by Conway & Koshland (1968) to be above 10^{11} M^{-1}, while the latter was found to be 2×10^5 M^{-1} (Eby & Kirtley, 1976). Other coenzyme fragments were also found to have lower binding constants to dehydrogenases compared with the intact coenzyme. Thus, the nicotinamide ring, apart from its central role in the chemical reaction catalyzed by the enzyme, also actively participates in the binding of the coenzyme.

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