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Relationship between Fluorescence and Conformation of ϵNAD$^+$ Bound to Dehydrogenases†

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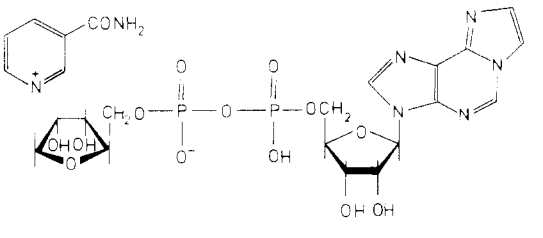
ABSTRACT: This work reports on the interaction of the fluorescent nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵNAD$^+$) with horse liver alcohol dehydrogenase, octopine dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase from different sources (yeast, lobster muscle, and rabbit muscle). The coenzyme fluorescence is enhanced by a factor of 10–13 in all systems investigated. It is shown that this enhancement cannot be due to changes in the polarity of the environment upon binding, and that it must be rather ascribed to structural properties of the bound coenzyme. Although dynamic factors could also be important for inducing changes in the quantum yield of ϵNAD$^+$ fluorescence, the close similarity of the fluorescence enhancement factor in all cases investigated indicates that the conformation of bound coenzyme is rather invariant in the different enzyme systems and overwhelmingly shifted toward an open form. Dissociation constants for ϵNAD$^+$–dehydrogenases complexes can be determined by monitoring the coenzyme fluorescence enhancement or the protein fluorescence quenching. In the case of yeast glyceraldehyde-3-phosphate dehydrogenase at pH 7.0 and t = 20° the binding plots obtained by the two methods are coincident, and show no cooperativity. The affinity of ϵNAD$^+$ is generally lower than that of NAD$^+$, although ϵNAD$^+$ maintains most of the binding characteristics of NAD$^+$. For example, it forms a tight complex with horse liver alcohol dehydrogenase and pyrazole, and with octopine dehydrogenase saturated by L-arginine and pyruvate. One major difference in the binding behavior of NAD$^+$ and ϵNAD$^+$ seems to be present in the muscle glyceraldehyde-3-phosphate dehydrogenase. In fact, no difference was found for ϵNAD$^+$ between the affinities of the third and fourth binding sites. The results and implications of this work are compared with those obtained recently by other authors.

Nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵNAD$^+$),[1] having the formula shown below, has a fluorescence maximum at around 410 nm when excited in the 305-nm region (Barrio *et al.,* 1972). It therefore offers the possibility of exploiting fluorescence techniques for studying the interaction between dehydrogenases and the oxidized form of the coenzyme. Moreover, since the fluorescence signal is localized on the adenine ring, information on the involvement of the adenosine moiety in the coenzyme binding can possibly be obtained. Our laboratory has al-



ϵNAD

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[1] Abbreviations used are: ϵNAD$^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid.

ready issued preliminary reports on the interaction between ϵNAD$^+$ and dehydrogenases (Bonner *et al.*, 1973; Baici and Luisi, 1974; Baici *et al.*, 1974). One of these reported on the interaction between octopine dehydrogenase and ϵNAD$^+$ (Baici *et al.*, 1974).

Although the affinity of this coenzyme analog for the enzyme was smaller than that of NAD$^+$, the enzyme was shown to have the same turnover number with the two compounds. Upon binding to the enzyme, the fluorescence of ϵNAD$^+$ had an ~10-nm blue shift and increased in intensity by a factor of ~13. Since this enhancement was considered too large to be ascribed solely to a change of the microenvironment (from water solution to hydrophobic protein regions), we suggested that it could be due to a conformational change of the coenzyme molecule. In particular, since there is some evidence to suggest that ϵNAD$^+$ and analogous compounds exist in water solution in the form of a stacked conformation (Barrio *et al.*, 1973; Scott *et al.*, 1970; Oppenheimer *et al.*, 1971), it was proposed that the fluorescence enhancement of ϵNAD$^+$ was caused by the shift of the conformational equilibrium to an open conformation upon binding. One of the main purposes of the present paper is to further discuss this possibility.

Also of particular interest is the fact that the binding of ϵNAD$^+$ to dehydrogenases can be followed by monitoring both the quenching of the protein fluorescence and the enhancement of the coenzyme fluorescence. One can therefore raise the question whether the binding plots obtained *via* the two methods are equal. The different affinity of NAD$^+$ and ϵNAD$^+$ for the various dehydrogenases will also be discussed.

The interaction of ϵNAD$^+$ with enzymes has been the subject of recent investigations in other laboratories. Lee and Everse (1973) consider the relation between intramolecular folding of ϵ-dinucleotides and quantum yield, as well as the quenching of the protein fluorescence upon coenzyme binding. Schlessinger and Levitzki (1974) consider in detail the binding of ϵNAD$^+$ to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by different spectroscopic techniques, with particular emphasis on the subunit cooperative interactions. More detailed references to these authors' data will be considered later on in the present paper.

Experimental Section

Materials

Preparation of ϵNAD$^+$ and 5'-ϵAMP. ϵNAD$^+$ was synthetized according to Barrio *et al.* (1972). However, the occasional presence of a yellow contaminant at the final stage in some of our preparations induced us to modify somewhat this procedure. After treating 1 mmol of NAD$^+$ (Calbiochem Grade A) with 20 ml of 1.5–2 M aqueous chloroacetaldehyde, the mixture was decolorized with charcoal and evaporated to about 4 ml under reduced pressure at 25°. After adjustment to pH 6.0 with NaOH, the solution was applied to a Dowex (1 × 1) column (Cl^- form), 30 × 2 cm, which was then washed with water. Elution was carried out with a 0–0.3 M LiCl gradient. Fractions having a ratio of 265 to 275 nm absorbance of 1.19 were pooled, and then evaporated under reduced pressure at 25° to 2 ml. The concentrate was then chromatographed through a G-15 Sephadex column (100 × 2 cm) (eluting with water) in order to eliminate LiCl. After pooling the fractions containing the product, ϵNAD$^+$ was precipitated with acetone and dried under vacuum. The approximate yield was 20%.

5'-ϵAMP was prepared according to Secrist *et al.* (1972). The ratio of 265 to 275 nm absorbance of our preparations was 1.0 in aqueous buffered solution at pH 7.0. *Anal.* Calcd for $C_{23}H_{27}N_7O_{14}P_2 \cdot 3H_2O$ (ϵNAD$^+$): C, 37.25; H, 4.49; N, 13.22. Found: C, 37.55; H, 4.43; N, 13.26. *Anal.* Calcd for $C_{12}H_{14}N_5O_7P \cdot H_2O$ (5'-ϵAMP): C, 37.02; H, 4.14; N, 17.99. Found: C, 36.91; H, 4.10; N, 17.89.

Both ϵNAD$^+$ and 5'-ϵAMP were shown to be pure by thin-layer chromatography (Eastman chromagram cellulose sheets) using the solvent mixture isobutyric acid–NH_4OH–water (75:1:24, v/v/v) suggested by Secrist *et al.* (1972). Concentrations were determined at pH 7.0 using extinction coefficients at 265 nm of 1.0×10^4 and 6.0×10^3 M^{-1} cm^{-1} for ϵNAD$^+$ and 5'-ϵAMP, respectively.

Enzymes. Octopine dehydrogenase (EC 1.5.1.15) was prepared by Miss M. O. Doublet at the Collège de France in Paris (Biochimie Cellulaire) and assayed according to van Thoai *et al.* (1969). Enzyme preparations having an activity between 90 and 100% were used (referred to a turnover number of 1700 (micromoles of NADH/minute per milligram of enzyme) at pH 6.6, t = 33°). The enzyme had A_{280}/A_{260} ratios of 1.9–2.0.

Horse liver alcohol dehydrogenase (EC 1.1.1.1) was purchased from Boehringer and purified as previously described (Luisi and Favilla, 1972). Enzyme activity was determined with the pyrazole method (Theorell and Yonetani, 1963) and found to be in the range 90–100%. In some experiments (for instance enzyme + ϵNAD$^+$ in the higher alkaline pH range) it has been necessary to eliminate traces of alcohol impurities which usually accompany the enzyme preparations. This has been done as recently described (Luisi and Bignetti, 1974).

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). The yeast enzyme was a gift of Professor K. Kirschner (Biozentrum, Basel) and was assayed as described by Kirschner and Voigt (1968). The enzyme activity was 148 units/mg. The A_{280}/A_{260} ratio of the apoenzyme was 2.10. The lobster (*Palinurus vulgaris*) muscle enzyme was a gift from Dr. G. L. Rossi in Parma (Institute of Molecular Biology) and was prepared by R. Verne according to the method of Allison and Kaplan (1964). When the activity was assayed following the method of Ferdinand (1964), an activity of 140 units/mg was found. When the enzyme was assayed according to Kirschner and Voigt (1968), we obtained 86 units/mg.

The rabbit muscle enzyme was the commercial product of Boehringer and activity was 90 units/mg when assayed according to Ferdinand (1964). Some experiments were repeated with a crystalline rabbit muscle preparation obtained in the laboratory of Dr. Hollaway (University College, London) and very kindly put at our disposal. This enzyme had an activity of 180 units/mg when assayed according to Ferdinand (1964).

All muscle enzymes were treated with charcoal as described by Murdock and Koeppe (1964). Experiments were performed with enzyme preparations having an A_{280}/A_{260} ratio of 1.5, indicating about 2 mol of NAD$^+$ bound per tetramer.

Phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) from *Crotalus terr. terr.* was the commercial product from Boehringer, and was used in microliter amounts without further purification (see Figure 1).

Methods

Fluorescence Measurements. The technique for evaluat-

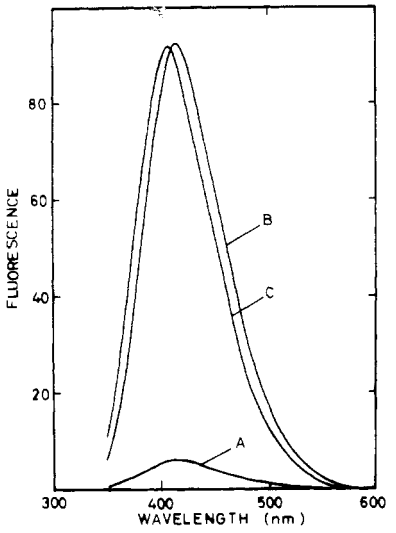

FIGURE 1: Emission spectra of ϵNAD^+ in 0.1 M sodium phosphate buffer (pH 7.0, temperature 25 ± 1°): (A) 5.4 μM ϵNAD^+; (B) 5.4 μM ϵNAD^+ after enzymatic hydrolysis with phosphodiesterase from *Crotalus* venom; (C) 5.4 μM ϵNAD^+ stoichiometrically bound in the horse liver alcohol dehydrogenase–pyrazole complex (enzyme 36 μN, pyrazole 1.3 mM). 5′-ϵAMP at a concentration of 5.4 μM gives rise to an emission spectrum identical with B. The spectra are uncorrected; excitation, 320 nm.

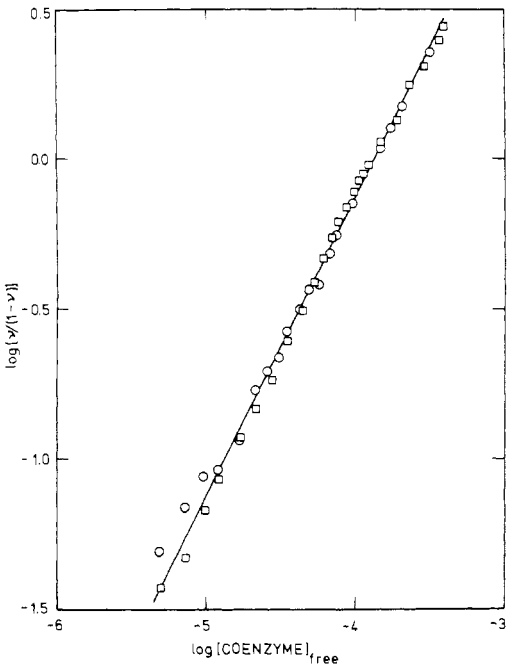

FIGURE 2: Hill plots for the binding of ϵNAD^+ to glyceraldehyde-3-phosphate dehydrogenase from yeast: (○) binding followed by monitoring the ϵNAD^+ fluorescence enhancement, excitation 325 nm and emission 410 nm; (□) binding followed by monitoring the protein fluorescence quenching, excitation 280 nm and emission 325 nm. Conditions were 4.8 μN enzyme in 0.1 M sodium phosphate buffer containing 5 mM EDTA and 0.025 mM Cleland's reagent, pH 7.0, temperature 20 ± 1°.

ing the binding constants from protein fluorescence quenching and/or coenzyme fluorescence enhancement has been described in previous papers (Luisi *et al.*, 1973; Baici *et al.*, 1974). Basically, the molar fraction of enzyme-bound sites, $\nu_i = [ES_i]/[E_{tot}]$, is correlated to the fluorescence changes ΔF_i through the equation $\nu_i = \Delta F_i/\Delta F_{max}$, where ΔF_{max} is the maximal fluorescence quenching (enhancement), namely that obtained by extrapolation to infinite ligand concentration. Fluorescence inner filter effects are corrected according to Parker (1968) with an appropriate computer program, which also gives the average value of the binding constant (in the case of linear plots) *via* a linear regression. An example of the binding plots is given in Figure 2. All enzyme concentrations are referred to the active-site concentration, which is given throughout this paper as N (normality). Quartz cuvets with a 1–2-mm light path were used.

Quantum yields (see Table I for some actual calculations) were measured using quinine sulfate in 0.1 N H_2SO_4 as a reference and assuming the quantum yield of this compound to be 0.55 (Dawson and Windsor, 1968). Scott *et al.* (1970) report that the quantum yield of quinine sulfate in 0.1 N H_2SO_4 is 0.70, but this value is referred to corrected fluorescence spectrum. Since we refer to uncorrected spectra, we prefer to use the value 0.55 as a reference. Moreover, recent data by Gelernt *et al.* (1974) indicate a value of 0.561 also for the absolute quantum yield of this compound.

In evaluating the blue shift of the fluorescence maximum of bound coenzyme, it is important to correct for the shorter wavelength band arising from excitation light scattering. This superposition gives an apparently larger blue shift. Corrections have been made with the help of a DuPont Model 310 curve resolver. Corrections for the base line (particularly important for a precise evaluation of the quantum yields) and determination of peak areas were also directly carried out with this analog computer (see Table I).

Enzymatic Hydrolysis of ϵNAD^+. Phosphodiesterase (10 μg) from *Crotalus terr. terr* (EC 3.1.4.1) was added to 0.5 ml of 5.4 μM ϵNAD^+ solution (0.1 M phosphate buffer, pH 7.0, t = 25°). The time course of the reaction was measured at 410 nm (excitation at 320 nm) by monitoring the fluorescence intensity increase. The spectrum of Figure 1B was recorded at the end of the reaction, which is completed in about 45 min.

Results

Enhancement of ϵNAD^+ Fluorescence upon Binding to Dehydrogenases. Table II reports the fluorescence enhancement for ϵNAD^+ bound to octopine dehydrogenase, horse liver alcohol dehydrogenase, and yeast, rabbit muscle, and lobster muscle glyceraldehyde-3-phosphate dehydrogenase. The case of octopine dehydrogenase has already been reported (Baici *et al.*, 1974) but we thought it worthwhile to compare it with the other dehydrogenases.

The most relevant feature of Table II is the constancy of the enhancement factor in all systems investigated; the quantum yield of bound ϵNAD^+ increases by a factor of 10–13 with respect to that of the free coenzyme, independent of the particular enzyme.

Schlessinger and Levitzki (1974) have recently reported on the interaction between ϵNAD^+ and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. Contrary to us, they find that the coenzyme fluorescence of the bound ϵNAD^+ is enhanced by about a factor of three. The reason for this difference will be considered later on, in relation to the value of the binding constants.

Another relevant feature of ϵNAD^+ fluorescence is shown in Figure 1. It is known that phosphodiesterase hydrolyzes NAD^+ giving NMN and AMP. In the case of ϵNAD^+, as reported by Barrio *et al.* (1972), this reaction is accompanied by an increase of the 410-nm fluorescence, without any shift of the fluorescence maximum. The signifi-

Table I: Quantum Yield of ϵNAD+ Bound to Dehydrogenases.

Composition of the System	Buffer and Temp (°C)	$[\epsilon NAD^+]_{free}$[a] (μM)	Relative Areas[b] Mixture	Total ϵNAD+	Free ϵNAD+	Quantum Yield of Bound ϵNAD+ (±0.02)[c] Uncorrected	Corrected
Liver ADH[d] (36 μN), pyrazole (1.3 mM), ϵNAD+ (5.4 μM)	0.1 M phosphate (pH 7.0), $t = 25$	~0	100.0	7.5	0	0.34	0.43
ODH[e] (13.8 μN), L-arginine (0.02 M), pyruvate (0.02 M), ϵNAD+ (4.58 μM)	0.1 M phosphate (pH 7.0), $t = 25$	1.46	100.0	11.0	3.5	0.33	0.42
Yeast GAPDH[f] (103 μN), ϵNAD+ (255 μM)	0.1 M phosphate–5 mM EDTA–0.025 mM DTT[h] (pH 7.0), $t = 20$	203	100.0	29.9	23.8	0.32	0.41
Lobster GAPDH[f] (64 μN)[g] ϵNAD+ (117 μM)	0.1 M phosphate–5 mM EDTA (pH 7.0), $t = 25$	98.5	100.0	36.0	30.3	0.32	0.41
Rabbit GAPDH[f] (152 μN),[g] ϵNAD+ (15 μM)	0.1 M phosphate–0.01M EDTA (pH 7.5), $t = 25$	5.2	100.0	13.0	4.5	0.29	0.37

[a] Calculated on the basis of the binding constants of Table IV. [b] Corrected for inner filter effects. [c] This is given by: [(area mixture − area free ϵNAD+)/(area total ϵNAD+ − area free ϵNAD+)] × 0.026, where 0.026 is the *uncorrected* quantum yield of ϵNAD+ in 0.1 M phosphate buffer (pH 7.0). The corrected quantum yield of ϵNAD+ on the basis of which the last column has been computed is 0.033 in the same conditions (see Experimental Section). [d] Alcohol dehydrogenase. [e] Octopine dehydrogenase. [f] Glyceraldehyde-3-phosphate dehydrogenase. [g] This is the total *active*-site concentration. Note, however, that the enzyme used in these experiments contained about 2 mol of NAD+ bound/tetramer and the calculation of the binding constants has been therefore carried out assuming that only one-half of the enzyme active sites are available for ϵNAD+ (see Experimental Section). [h] DTT, dithiothreitol.

cant point here is that the results in Figure 1 and Table II suggest that the enhancement factor obtained when ϵNAD+ is cleaved enzymatically is the same as that observed with dehydrogenase-bound ϵNAD+. As far as the fluorescence intensity is concerned, therefore, the cleaved ϵAMP moiety is equivalent to the ϵAMP moiety of the whole coenzyme bound. The enzymatic hydrolysis has been considered also by Lee and Everse (1973). In the case of ϵ-FAD, they found that the addition of snake venom phosphodiesterase results in an increase in the fluorescence of the ϵ-adenine as well as that of the flavine moiety, with values approaching those expected for the corresponding mononucleotides.

It seemed relevant, at this point, to ask what is the effect of changes in the microenvironment around the ϵAMP fluorofor on the fluorescence intensity. This question has some relevance, since upon binding to a protein a fluorofor usually shows a blue shift in its fluorescence maximum and an increase of its quantum yield (see, for instance, Stryer, 1965; Edelman and McClure, 1968; Kenner and Aboderin, 1971).

Table III shows that on going from buffer at pH 7.0 to 80% dioxane, 5′-ϵAMP undergoes practically no fluorescence change, whereas the fluorescence of ϵNAD+ increases by a factor of 2.6. Apart from the question of the different behavior of 5′-ϵAMP and ϵNAD+ these data show that the enhancement of the fluorescence of 5′-ϵAMP due to a decreased polarity in the microenvironment is very low.

The blue shift of the fluorescence maximum going from buffer at pH 7.0 to 20% water–80% dioxane is around 5 nm for 5′-ϵAMP and 10 nm for ϵNAD+ (both compounds are insoluble in pure dioxane). In all enzyme systems investigated, we observed a blue shift of the fluorescence maximum of bound ϵNAD+. This shift is around 10 nm for octopine dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, and only 5–6 nm for horse liver alcohol dehydrogenase. A more precise determination of the spectral change is not possible with our uncorrected fluorometer.

Table II: Fluorescence Properties of ϵNAD+ in Various Systems.

	Process	Fluorescence Enhancement[a]	Blue Shift (±2 nm)
ϵNAD+ →	Bound to LADH[b]	13.1 ± 1	6
	Bound to ODH[c]	12.7 ± 1	10
	Bound to yeast GAPDH[d]	12.3 ± 1	10
	Bound to lobster GAPDH[d]	12.3 ± 1	10
	Bound to rabbit GAPDH[d]	11.2 ± 1	10
	Cleaved into NMN + 5′-ϵAMP	13.5 ± 1	0
	From H_2O to 80% dioxane	2.9 ± 0.2	10

[a] Calculated on the basis of the ratio of the areas under the peaks before and after the indicated process. [b] Alcohol dehydrogenase from horse liver. [c] Octopine dehydrogenase. [d] Glyceraldehyde-3-phosphate dehydrogenase.

Table IV shows the dissociation constants of ϵNAD+ from the various enzymes, together with the dissociation constants of NAD+ reported in the literature. Note that the affinity of ϵNAD+ is generally lower than that of NAD+, and that a greater NAD+ affinity corresponds to a greater ϵNAD+ affinity. For octopine dehydrogenase and yeast

Table III: Fluorescence of ϵNAD$^+$ and 5′-ϵAMP in Various Media.

Solvent	Rel. Fluorescence[a]	
	ϵNAD$^+$	5′-ϵAMP
0.1 M phosphate, pH 7.0	1.00	1.00
20% 1,4-dioxane in water	1.52	0.97
40% 1,4-dioxane in water	2.12	1.07
50% 1,4-dioxane in water	2.30	1.06
60% 1,4-dioxane in water	2.48	1.08
80% 1,4-dioxane in water	2.64	1.08

[a] The data are expressed as the ratio of the areas under the peaks, with the area of the compound in buffer at pH 7.0 being unity as an arbitrary reference; indeterminateness ±5%. Concentration was 1×10^{-4} M of ϵNAD$^+$ and 5′-ϵAMP in the indicated solvent; compositions of the water–dioxane mixtures are given as w/w %.

glyceraldehyde-3-phosphate dehydrogenase, that we investigated in detail, binding plots for ϵNAD$^+$ were linear. For the last enzyme, the absence of cooperativity in the coenzyme binding at pH 7 and $t = 20°$ had been already pointed out by Kirschner *et al.* (1966). Figure 2 shows the Hill plots for the binding of ϵNAD$^+$ to yeast glyceraldehyde-3-phosphate dehydrogenase, characterized by a Hill coefficient equal to 1. ϵNAD$^+$ and ϵNADH are able to bind to chicken muscle lactate dehydrogenase, as shown by Lee and Everse (1973). In particular, they report that the affinity of ϵNAD$^+$ is larger than that of NAD$^+$, whereas the affinity of ϵNADH is smaller than that of NADH.

As it is well known, the binding of NAD$^+$ to rabbit and lobster muscle glyceraldehyde-3-phosphate dehydrogenase is characterized by different sets of dissociation constants (see, for instance, De Vijlder and Slater, 1968; De Vijlder *et al.*, 1969). After the very tight binding of the first two sites, further binding proceeds with decreased affinity, and K_d values for the third and fourth sites are different. Schlessinger and Levitzki (1974) found that the binding of ϵNAD$^+$ also proceeds in a similar manner. In particular, they report that the affinities to the third and fourth sites differ by almost an order of magnitude. On the contrary, we have been unable to find a sizable discontinuity in the binding plot of ϵNAD$^+$ for the third and fourth sites. We obtain the same results in the buffer used by Schlessinger and Levitzki (1974) (pH 7.5, 0.05 M Hepes + 0.01 M EDTA).

It is difficult at the present to account for this difference, as well as for the aforementioned difference in the quantum yield of bound ϵNAD$^+$. Among the possible factors, one may consider that the high enzyme concentration used by Schlessinger and Levitzki in some of their binding experiments (see, for example, their Figure 2) might cause difficulties in exactly evaluating a small K_d value. The different evaluation of binding data may also be of some importance. For example, when we build a double reciprocal plot from Schlessinger and Levitzky's data (their insert in Figure 2) we obtain a value for the spectroscopic parameter (ΔA_{340}) corresponding to 100% saturation which is 10–15% higher than that indicated by these authors in the Figure 2 insert. Scatchard plots built on the basis of this new ΔA_{340} maximal value yield practically a straight line in the saturation region between $\nu = 0.5$ and $\nu = 1.0$, and with a higher K_d value than that given by these authors for their K_3.

The difference between Schlessinger and Levitzki's and our data might also be due to more subtle factors. For example, for ϵNAD$^+$ binding studies we add ϵNAD$^+$ to E(NAD)$_2$, whereas they, more properly, do so with E(ϵNAD)$_2$.

We already reported that ϵNAD$^+$ binding to octopine dehydrogenase is accompanied by a quenching of the protein fluorescence (Baici *et al.*, 1974). We found the same for all other systems investigated in this paper.

Table IV: Dissociation Constants for ϵNAD$^+$ and NAD$^+$.

Enzyme	Buffer and Temp (°C)	K_d (μM)		Ref for NAD$^+$ Data[a]
		ϵNAD$^+$	NAD$^+$	
ODH[d] saturated by L-arginine + pyruvate	0.1 M phosphate (pH 7.0), $t = 25$	5.0	1.1	Luisi *et al.*, 1973
Horse liver ADH[e] saturated by pyrazole	0.1 M phosphate (pH 7.0), $t = 25$	<1	0.1	Theorell and Yonetani, 1963
Yeast GAPDH[f]	0.1 M phosphate–5 mM EDTA–0.025 mM DTT[g] (pH 7.0), $t = 20$	135	4.2	Velick *et al.*, 1971
Lobster muscle GAPDH[f]	0.1 M phosphate–5 mM EDTA (pH 7.0), $t = 25$	74[c]	0.6; 13[b]	De Vijlder *et al.*, 1969
Rabbit muscle GAPDH[f]	0.05 M Hepes–0.01 M EDTA (pH 7.5), $t = 25$	35[c]	4; 35[b]	De Vijlder and Slater, 1968

[a] Data for NAD$^+$ refer to conditions of temperature and pH similar but never identical with our conditions for ϵNAD$^+$. [b] Numbers refer, respectively, to the third and fourth binding constants. [c] As mentioned in the Results Section, we found only one dissociation constant for the third and fourth binding sites. Schlesinger and Levitzki (1974) found for the binding of ϵNAD$^+$ to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase K_d values of 3.1 and 17.5 μM, respectively, for the third and fourth binding sites. [d] Octopine dehydrogenase. [e] Alcohol dehydrogenase. [f] Glyceraldehyde-3-phosphate dehydrogenase. [g] DTT, dithiothreitol.

Discussion

The blue shift in the maximum of ϵNAD$^+$ fluorescence upon binding to dehydrogenases (Table II) indicates that the adenine moiety "senses" hydrophobic regions, *i.e.*, it is directly involved in the coenzyme binding. This interpretation is supported by the lack of blue shift upon enzymatic cleavage of ϵNAD$^+$. The magnitude of the blue shift, being different for different enzymes, probably suggests the obvious: that the environment in the adenine binding site is different for different proteins.

Since binding of a fluorofor to a hydrophobic protein region is often accompanied by fluorescence enhancement, our observed equality between ϵNAD$^+$ fluorescence enhancement when bound to dehydrogenases and when enzymatically cleaved might appear surprising. This equivalence along with the observation that 5′-ϵAMP fluorescence is found rather insensitive to changes in hydrophobicity (Table III) and to changes in pH above 7 (Spencer *et al.*, 1974) leads us to conclude that changes in the solvent microenvironment of the ϵ-adenine moiety are not an important factor in our observed fluorescence enhancement. This consideration, together with the fact that the enhancement of ϵNAD$^+$ is the same or very similar in different enzyme systems (Table I), suggests that this phenomenon reflects an intrinsic structural property of the coenzyme.

Spectroscopic data indicate that NADH, ϵNAD$^+$, and similar compounds assume stacked conformations in aqueous solution that result in intramolecular quenching of the nucleotide fluorescence (Secrist and Leonard, 1972; Barrio *et al.*, 1973; Oppenheimer *et al.*, 1971; Scott *et al.*, 1970). When this is considered in light of the equality between the fluorescence enhancement of ϵNAD$^+$ upon enzymatic cleavage and that obtained for bound ϵNAD$^+$ (Figure 1), one could conclude that the ϵNAD$^+$ fluorescence enhancement upon binding is due to an opening of the stacked coenzyme conformation. The mechanism producing such an enhancement would be in both cases (enzymatic cleavage and binding) the formation of an adenosine moiety, which is nonstacked with the nicotinamide moiety.

Consider also that we know from X-ray data that coenzyme binds in an open conformation to lobster muscle glyceraldehyde-3-phosphate dehydrogenase (Buehner *et al.*, 1973) and horse liver alcohol dehydrogenase (Bränden *et al.*, 1973), as well as to other dehydrogenases (Adams *et al.*, 1973; Webb *et al.*, 1973). It therefore seems reasonable to us to use the fluorescence data of Table I to suggest that coenzyme binds in an open conformation in all enzyme systems investigated in this work.

Lee and Everse (1973) have recently investigated the conformation of ϵNAD$^+$ in solution using nmr techniques, concluding that only ~40% of the coenzyme is present in a stacked conformation. On the basis of this finding, the authors concluded that other effects aside from intramolecular stacking should be responsible for the low quantum yield of free ϵNAD$^+$ as compared to 5′-ϵAMP. They assume that intramolecular dynamic quenching is such an effect. However, their hypothesis, as stated, relies on the fluorescence lifetime of ϵNAD$^+$ being relatively long (~23 nsec) compared to its folding and unfolding rate (5 nsec); yet, Barrio *et al.* (1972) state that the fluorescence lifetime is shorter than 23 nsec and Schlessinger and Levitzki (1974) mention a value of only 4 nsec.

In view of this, it is difficult to evaluate at present the importance of dynamic quenching on the fluorescence quantum yield of ϵNAD$^+$ in solution. To the extent that such a dynamic quenching would be decreased upon binding, enhancement of ϵNAD$^+$ fluorescence becomes less a direct measure of the conformational change.

Consider also that an enhancement factor lower than 10–13, possibly found in some new enzyme complex, could not be taken to directly indicate that coenzyme is bound in a folded conformation. In fact, the lower quantum yield could be caused by the interaction of the ϵ-adenine moiety with a quenching group at the enzyme site. This last point is also made by Lee and Everse (1973) on the basis of preliminary data concerning the binding of ϵNADH to chicken muscle lactate dehydrogenase.

It is relevant at this point to ask the question whether it is reasonable to transfer directly to NAD$^+$ the information obtained for ϵNAD$^+$. It should be borne in mind that in the case of octopine dehydrogenase we obtain the same turnover number for both coenzymes (Baici *et al.*, 1974); the same holds for yeast alcohol dehydrogenase and lactate dehydrogenase (Lee and Everse, 1973). Also note that ϵNAD$^+$ has several characteristic features of NAD$^+$; for instance it forms a tight complex with horse liver alcohol dehydrogenase and pyrazole, and with octopine dehydrogenase when this is saturated by L-arginine and pyruvate. Furthermore, when ϵNAD$^+$ binds to glyceraldehyde-3-phosphate dehydrogenase, the Racker band is formed (Schlessinger and Levitzki, 1974).

It is interesting to compare the fluorescence properties of ϵNAD$^+$, in which the fluorofor is localized on the adenine ring, with those of NADH, in which the fluorofor is localized on the nicotinamide ring. Whereas ϵNAD$^+$ fluorescence intensity seems to respond very similarly to the different dehydrogenases, at least in the systems investigated in this paper, NADH fluorescence intensity shows a less regular pattern. For instance, it is enhanced in horse liver alcohol dehydrogenase (Winer and Theorell, 1960), octopine dehydrogenase (Luisi *et al.*, 1973), and many other dehydrogenases, whereas it is quenched in muscle glyceraldehyde-3-phosphate dehydrogenase (Velick, 1958) and in some ternary complexes of lactate dehydrogenase (Südi, 1974). In passing, perhaps it is of interest to note that in the enzymatic hydrolysis of NADH by phosphodiesterase we observed no fluorescence enhancement—on the contrary, about 30% quenching was observed.

This comparison suggests that different fluorofors can be affected in quite different ways by the same structural changes, so that no *a priori* generalization is possible concerning the use of fluorescence intensity for obtaining information about the conformational equilibria. Although aware of these limits, we conclude that the ϵ-adenine moiety is a useful reporter in binding studies and that the fluorescence enhancement we observed can be taken to suggest unfolding of the coenzyme when bound, though other factors, which could only be resolved by other spectroscopic techniques, could also affect the fluorescence.

The analysis of the dissociation constants of ϵNAD$^+$ is of some interest. The decreased affinity and the blue shift of the bound ϵNAD$^+$ fluorescence maximum relative to NAD$^+$ indicate that the adenine ring binds to the protein and that this binding sub-site is sensitive to the chemical modification introduced into the adenine ring. This chemical modification is, however, not able to modify the affinity pattern relative to NAD$^+$. For example, the high affinity in the complexes of horse liver alcohol dehydrogenase or octopine dehydrogenase is maintained.

The large difference in the binding constants of NAD$^+$

and ϵNAD^+ in yeast glyceraldehyde-3-phosphate dehydrogenase is worth attention, particularly in view of the minor effect in the muscle enzymes. It is tempting to speculate that this reflects a different structure in the adenine binding subsite of the enzymes, but the confirmation of this hypothesis has to await the X-ray structure. It is also interesting to remark that in yeast glyceraldehyde-3-phosphate dehydrogenase the binding of ϵNAD^+ at pH 7.0 and $t = 20°$ is linear just as that of NAD^+. It will be interesting to investigate the binding of ϵNAD^+ at pH 8.5, under the conditions where NAD^+ binding shows a sigmoid behavior (Kirschner *et al.,* 1966), and ask the question whether the chemical modification on the adenine ring modifies the subunit interaction in the enzyme. It is also worthwhile to repeat that in the case of the rabbit muscle enzyme we do not find for ϵNAD^+ any difference between the third and fourth binding sites. The same holds for the lobster muscle enzyme. This difference in behavior between NAD^+ and ϵNAD^+ certainly warrants more detailed investigation.

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