8, 24 (1962); (d) J. Baddiley, W. Frank, N. A. Hughes, and J. Wieczorkowski, *J. Chem. Soc.*, 1999 (1962).
(4) (a) R. T. Borchardt and Y. S. Wu, *J. Med. Chem.*, **18**, 300 (1975); (b) R. T. Borchardt, J. A. Huber, and Y. S. Wu, *J. Org. Chem.*, **41**, 565 (1976); (c) R. T. Borchardt and Y. S. Wu, *J. Med. Chem.*, **19**, 197 (1976).
(5) (a) G. A. Jamieson in "Synthetic Procedures in Nucleic Acid Chemistry", Vol. 1, W. W. Zorbach and R. S. Tipson, Ed., Interscience, New York, N.Y., 1968, pp 176 and 215; (b) R. T. Borchardt, Y. S. Wu, J. A. Huber, and A. F. Wycpalek, *J. Med. Chem.*, **19**, 1104 (1976).
(6) R. T. Borchardt and Y. S. Wu, *J. Med. Chem.*, **17**, 862 (1974).
(7) J. Baddiley, *J. Chem. Soc.*, 1348 (1951).
(8) R. S. Schmidt, U. Schloz, and D. Schwille, *Chem. Ber.*, **101**, 590 (1968).
(9) R. E. Harmon, C. V. Zenarosa, and S. K. Gupta, *Chem. Ind. (London)*, 1141 (1969).
(10) (a) P. A. Levene, L. W. Bass, and H. S. Simms, *J. Biol. Chem.*, **70**, 229 (1926); (b) P. A. Levene, H. S. Simms, and L. W. Bass, *ibid.*, **70**, 243 (1926); (c) R. M. Izatt, L. D. Hansen, J. H. Rytting, and J. J. Christensen, *J. Am. Chem. Soc.*, **87**, 2760 (1965); (d) J. J. Christensen, J. H. Rytting, and R. M. Izatt, *ibid.*, **88**, 5105 (1966); (e) R. M. Izatt, J. H. Rytting, L. D. Hansen, and J. J. Christensen, *ibid.*, **88**, 2641 (1966).
(11) P. Mamalis and H. N. Ryden, *J. Chem. Soc.*, 1049 (1955).
(12) (a) W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, N.Y., 1969; (b) C. A. Bunton and V. J. Shiner, Jr., *J. Am. Chem. Soc.*, **83**, 42 (1961).
(13) W. H. Saunders and D. H. Edlson, *J. Am. Chem. Soc.*, **82**, 138 (1960).
(14) T. M. Chu, M. F. Mallette, and R. O. Mumma, *Biochemistry*, **7**, 1399 (1968).

Variation of the Linear Polarization Across the Emission Band of Nicotinamide 1,N^6-Ethenoadenine Dinucleotide Bound to Dehydrogenases

A. Gafni,* J. Schlessinger,† and I. Z. Steinberg

Contribution from the Department of Chemical Physics, Weizmann Institute of Science, Rehovot, Israel. *Received December 5, 1977*

Abstract: The linear polarization of the fluorescence of nicotinamide 1,N^6-ethenoadenine dinucleotide bound to several dehydrogenases studied varies significantly with emission wavelength. This spectral behavior is different for the different enzymes. No dependence of the linear polarization on the emission wavelength was observed for the free dinucleotide in glycerol solution. Similarly, the polarization spectrum of the excitation light of the complexes studied depends on the wavelength of the fluorescence selected for the measurements. The above findings are attributed to the fact that the transition responsible for the emission of the 1,N^6-ethenoadenine chromophore is weak. This transition thus "borrows" intensity from transitions involving higher electronic levels by vibronic coupling, thus conferring different polarization behavior to different vibronic transitions in the emission band. It is suggested that the sensitivity of the spectrum of the linear polarization across emission bands to the environment of the fluorophore in the case of weak transitions may be applied to the study of binding sites of biopolymers and other biological structures.

Introduction

1,N^6-Ethenoadenine and its derivatives are strongly fluorescent and have therefore been introduced as fluorescent probes in the study of nucleic acids and enzymes which require derivatives of adenine as substrates, cofactors, or effectors.[1-5] The 1,N^6-ethenoadenine derivatives retain a considerable fraction of the biochemical activity of the parent compounds in most of the cases studied;[1,2,4,5] the fluorescence properties of the etheno compounds are thus relevant to the specific binding sites of the macromolecules. It has been pointed out[5] that one of the important possibilities offered by these fluorescent compounds is the study of the linear polarization of the emitted light, which may be applied to the study of the rotational relaxation times of macromolecules of high molecular weight, owing to the relatively long fluorescence lifetime.

While a long lifetime of a fluorophore may permit wider application of its linear polarization in the study of macromolecules, it may, on the other hand, lead to serious complications of a fundamental nature in the behavior of the polarization of the fluorescence. The reason for this is that a long fluorescence lifetime of a fluorophore demonstrates that the electronic transition responsible for the emission is weak. In transitions of this kind the polarization properties in light absorption or emission depend not only on the electronic levels between which the transition takes place, but also on the particular vibrational levels of the ground and excited states which are involved in the absorption or emission at a specified region of the spectrum.[6-8] The polarization may consequently change across a spectral band which corresponds to a single electronic transition. Furthermore, the polarization may be sensitive to perturbations by the environment of the chromophore. Such behavior may be particularly pronounced if there is a strong electronic band located in the spectrum near the band under consideration.[6] It is thus obvious that the simplifications in the measurement and the interpretation of linear polarization which are permitted when the polarization is constant for every electronic transition cannot be utilized in the case of weak transitions, and one should be alert to possible complications which may arise because of the peculiarities of such transitions.

In recent studies of the *circular* polarization of the fluorescence of nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵ-NAD^+) bound to glyceraldehyde 3-phosphate dehydrogenase (GPDH), an anomaly was noted in the spectral behavior of the circular polarization across the emission band.[9] This anomaly was attributed to the fact that the transition involved is a weak one. It has thus become of interest to test whether the "weakness" of the transition is also manifested in the spectral behavior of the linear polarization of the transition. In the following we present the results of this study. As will be shown below, the linear polarization of the fluorescence of ϵ-NAD^+ may indeed vary significantly across the emission band and the spectrum of the linear polarization is affected significantly by the environment of the fluorophore. The implications and possible applications of these findings will be discussed.

† NIH, Bethesda, Md. 20014.

0002-7863/79/1501-0463$01.00/0

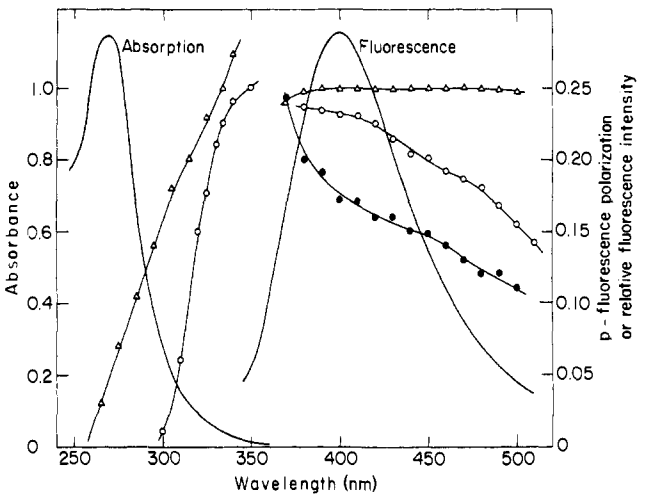

Figure 1. Spectroscopic results obtained for ε-NAD^+ in glycerol and when bound to LDH or to LADH. The absorption and emission spectra were obtained for a ~1.4×10^{-4} M solutio n in glycerol. The fluorescence was excited at 335 nm. (-Δ-Δ-), linear polarization of the fluorescence in glycerol. Emission at 420 nm was used to determine the dependence of the polarization on the wavelength of excitation. The linear polarization at different emission wavelengths was studied using 335-nm excitation. (-O-O-), linear polarization of fluorescence of LADH-ε-NAD^+- pyrazole ternary complex in 0.1 M phosphate buffer (pH 7.4). Concentrations follow: LADH, 5×10^{-5} M; ε-NAD^+, 5×10^{-5} M; pyrazole, 8×10^{-3} M. The linear polarization across the excitation spectrum was studied for the emission at 420 nm. The polarization spectrum across the emission band as shown in the figure is for 335-nm excitation and was computed from the experimentally obtained polarization spectrum, excited at 325 nm, in the following way. The angles, α, between the oscillators in absorption (at 325 nm) and emission at different wavlengths were calculated by means of eq 1. The angle, $\Delta\alpha$, between the absorption oscillators at 325 and 335 nm was calculated from the values of polarization at 420 nm excited by light of these two wavelengths. The values of $\alpha + \Delta\alpha$ were then used, in eq 1, to yield the desired p values for 335-nm excitation. (-●-●-), linear polarization of emission of LDH-ε-NAD^+-oxalate ternary complex in 0.1 M phosphate buffer (pH 7.4). Concentrations follow: LDH, 1.5×10^{-5} M; ε-NAD^+, 3×10^{-5} M; oxalate, 0.01 M. The excitation wavelength was 335 nm.

Materials and Methods

ε-NAD^+ and apoglyceraldehyde 3-phosphate dehydrogenase (apo-GPDH) were a gift from A. Levitzki, the Hebrew University, Jerusalem. Full details regarding the synthesis and the procedure of purification of the ε-NAD^+ were described elsewhere.[10] The complexes GPDH(ε-$NAD^+)_n$ (n = 1, 2, or 4) were dissolved in 0.05 M HEPES buffer, pH 7.5, containing 0.01 M EDTA. Protein concentration was 8×10^{-5} M. Horse liver alcohol dehydrogenase (LADH) and beef heart lactate dehydrogenase (LDH) were purchased from Boehringer Mannheim Co. as crystalline suspensions. The suspensions were centrifuged and the precipitated proteins dissolved in 0.1 M phosphate buffer of the desired pH and dialyzed against several fresh additions of the same buffer for 48 h. Enzyme concentrations were determined from the optical density at 280 nm.[11,12]

Absorption spectra were measured with a Zeiss Model PMQ II spectrophotometer. Corrected fluorescence spectra were obtained with a Hitachi Perkin-Elmer Model MPF-3 spectrofluorimeter. Fluorescence polarization spectra were obtained with an instrument built in this laboratory and described elsewhere.[13] This instrument is very similar to the one used in this laboratory to study circular polarization of luminescence.[14] To study the linear polarization of fluorescence the sample was excited by a beam of linearly polarized light and the intensity of the emitted light, modulated at twice the fundamental frequency of the light modulator, was detected. Some of the polarization spectra were also studied using an instrument of the type described by Weber and Babloužian[15] built by M. Shinitzky at our Institute. The fluorescence polarization, p, is given by $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, where $I_\parallel$ and $I_\perp$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular, respectively, to the direction of polarization of the exciting light. (The excitation light is linearly polarized, perpendicular to the plane defined by the directions of excitation and observation.)

All the measurements were done at room temperature (~23 °C).

Results

Figure 1 presents the absorption and fluorescence spectra of ε-NAD^+ in glycerol and the spectra of linear polarization across the absorption and emission of the coenzyme analogue dissolved in glycerol and bound to LADH in the ternary complex with pyrazole. The polarization of the emission of the LDH-ε-NAD^+-oxalate ternary complex is also shown. The polarization of fluorescence of ε-NAD^+ both in glycerol solution and when bound to LADH is seen to depend markedly on the excitation wavelength and increases rapidly toward the long-wavelength edge of the absorption spectrum. The linear polarization of fluorescence of ε-NAD^+ in glycerol was found to be independent of the emission wavelength while the polarization changes markedly across the emission spectra of ε-NAD^+ bound to LADH or to LDH. In the latter case the polarization changes more than twofold between 370 and 500 nm. Under the experimental conditions used the coenzyme analogue was fully bound to the dehydrogenases (>99% binding).[16,17] Thus the changes observed in the polarization are not due to the existence of unbound ε-NAD^+ in any of the samples studied. It has been shown that the fluorescence of ε-NAD^+ bound to LDH or to LADH decays according to a single exponential decay law which is independent of the degree of saturation of the binding sites by the coenzyme.[16] Thus, no heterogeneity of bound ε-NAD^+ molecules exists and the conclusion is reached that the large variation in polarization originates in a change in direction of the transition dipole moments involved in the emission at various wavelengths of the spectrum. Furthermore, the change in direction of the transition dipole moment with wavelength is markedly different in the case of LDH, LADH, and GPDH (see below), indicating that the environment of the fluorophore has a very pronounced effect on the transition dipole moments.

The linear polarization of the fluorescence emitted by ε-NAD^+ bound to GPDH at various degrees of saturation of the binding sites is shown in Figure 2. The excitation wavelength was 335 nm in all four cases. The linear polarization of fluorescence of these complexes also varies significantly across the emission spectrum. It reaches a maximal value at 420 nm and decreases by about 30% toward the long-wavelength edge of the emission spectrum. While the general shape of the polarization spectrum does not change as the number of ε-NAD molecules bound increases, the polarization decreases in value as the degree of occupation of binding sites increases. This change is largest between the complexes E(ε-$NAD^+)_1$ and E(ε-$NAD^+)_2$.

Discussion

Figure 1 shows that there is a dramatic change in the fluorescence polarization with the wavelength of excitation across the absorption band at 250-350 nm and that the values obtained for the polarization are relatively low, being, in all the cases studied, considerably smaller than the theoretical upper limit of 0.5. The latter observation is in good agreement with previously published results of the polarization of ε-NAD^+ fluorescence in very viscous media[10] and with results of polarized fluorescence decay measurements of ε-ADP and ε-ATP.[20] Partial depolarization is due to nonparallel absorbing and emitting oscillators rather than to a rotational relaxation effect. The dependence of p on excitation wavelength is not uncommon since an absorption spectrum frequently involves more than one electronic transition. It seems therefore clear from the polarization spectra that a second electronic transition contributes to the absorption on the long-wavelength side of

the main absorption band. This second transition is relatively weak and not evident in the absorption spectrum. This conclusion is in full agreement with the results of Secrist et al.,[5] who reported that the absorption band of ε-adenosine which involves the same electronic transition responsible for emission is located at about 300 nm with a rather low molar extinction coefficient of about 3000.

While the steep variation in p with excitation wavelength could be explained as due to overlap of absorption bands, a different origin must be sought for the variation of the polarization across the emission band of ε-NAD^+ bound to LDH or LADH, since only one electronic transition is usually involved in light emission from molecules in solution. This behavior cannot be attributed to heterogeneity in the system, since, even if one assumes that the population of molecules is heterogeneous, the polarization across the composite fluorescence band is still expected to be constant. This is so because for a well-behaved electronic transition the polarization of the fluorophore should be the same regardless of its environment, as long as the Brownian rotation is frozen in the time scale of the lifetime of the excited state. Moreover, it was concluded above that no heterogeneity of emitting ε-NAD^+ molecules exists in complexes of ε-NAD^+ with LDH or LADH. The change in polarization must, therefore, have its origin in the nature of the electronic transition involved in emission. In general, the polarization is expected to be constant throughout an electronic band only for an allowed electronic transition, characterized by a large oscillator strength. However, if the transition is weak, as is evidenced by a relatively small extinction coefficient or by a long emission lifetime, the polarization of the electronic transition is not necessarily constant across the band, since at each wavelength the transition may involve more than one transition dipole moment. The apparent emission oscillator is in this case the sum of several oscillators each with its own direction and intensity. The relative intensities of these components are, generally, wavelength dependent and hence the dependence of the apparent oscillator direction (and of the polarization) on wavelength follows. The spectral behaviour of the fluorescence polarization of ε-NAD^+ is therefore not surprising in view of the low extinction coefficient of the 300-nm absorption band and the relatively long fluorescence lifetime of this chromophore. Luisi et al.[17] found the fluorescence quantum yield of ε-NAD^+ in the ternary complex with LADH and pyrazole to be 0.43, and the fluorescence lifetime of ε-NAD^+ in the same complex has been recently found to be 28.6 ns.[16] These findings indicate a relatively long radiative lifetime of over 60 ns (the longer radiative lifetime quoted earlier for ε-NAD^+ [9] may need revision since the decay kinetics of ε-NAD^+ in aqueous solution is not monoexponential and precludes a straightforward evaluation of the radiative lifetime). It is therefore concluded that it is the low oscillator strength of the electronic transition which is responsible for the strong dependence of the fluorescence polarization on the emission wavelength and on the environment of the fluorophore. It is probable that the steep variation of p with the wavelength of excitation (especially at the long-wavelength edge of the absorption spectrum) originates partially from the same cause.

In view of the above, the lack of variation of p of ε-NAD^+ across its emission band in glycerol solution needs some discussion. It should be pointed out that this is not a coincidence due to excitation at a specific wavelength (335 nm in Figure 1), since excitation at another wavelength (305 nm) similarly yielded a flat dependence of p on emission wavelength. Thus, either the "borrowed" intensity in the case of ε-NAD^+ dissolved in glycerol is smaller than for ε-NAD^+ bound to the enzymes, or (what seems more likely) the "borrowed" intensity is polarized at larger angles relative to the S_1–S_0 transition in the bound than in the freely dissolved fluorophore (S_0 and S_1 denote the ground and first excited electronic singlet states, respectively, of the system). It may be noted that the fluorophore, when bound to the specific binding sites of the enzymes with relatively high energy, is probably strained to some extent. This may affect the symmetry properties of the vibrations, resulting in a different pattern of mixing with the higher electronic levels, or may result in a shift in the direction of polarization of the $S_1 \rightarrow S_0$ transition upon binding of the fluorophore to the enzymes. In spite of the constant value obtained for p for ε-NAD^+ in glycerol across the emission band, the relatively low value obtained for p (compared to the theoretical upper limit of 0.5) need not be surprising, since the excitation and emission do not involve the same vibrational levels of the electronic states S_0 and S_1. This is because absorption at the long-wavelength edge involves transitions from the lowest vibrational state of S_0 to excited vibrational states of S_1, whereas emission is from the lowest vibrational state of S_1 to excited vibrational states of S_0. The vibronic perturbation of S_1 is different from that of S_0 because of their difference in energy[6] and probably also because of a difference in molecular geometry in the ground and excited states as indicated by the appreciable Stokes shifts. The direction of the polarizations of the absorption and emission may thus be different, resulting in a value of p which is lower than the theoretical upper limit.

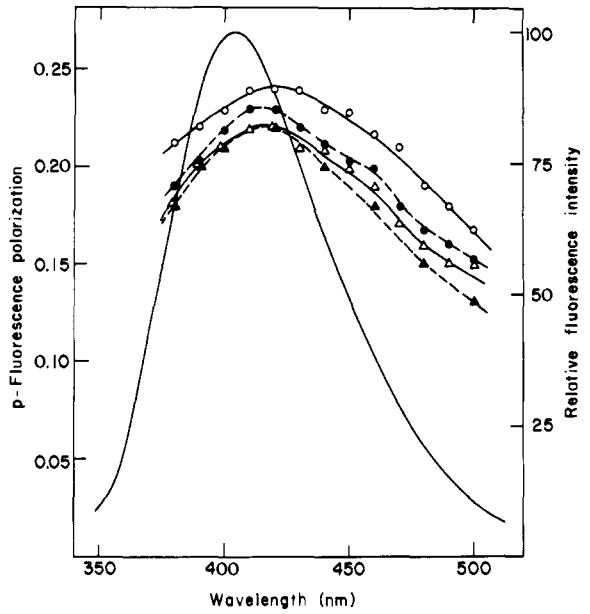

Figure 2. Linear polarization of fluorescence of GPDH(ε-NAD^+)$_n$ complexes in 50 mM HEPES buffer (pH 7.5) and 10 mM EDTA. GPDH concentration was 8×10^{-5} M in all cases and the excitation wavelength was 335 nm. ε-NAD^+ concentrations follow: (-O-O-), 8×10^{-5} M; (-●-●-), 16×10^{-5} M; (-Δ-Δ-), 24×10^{-5} M; (-▲-▲-), 32×10^{-5} M. In the complex GPDH(εNAD^+)$_4$ about 10% of the coenzyme analogue was not bound due to the smaller binding constant of the fourth ε-NAD^+ molecule to GPDH (10). However, since the fluorescence intensity of ε-NAD^+ is markedly enhanced upon binding (17), the contribution of free ε-NAD^+ to the total fluorescence is very small (about 1%), so that its effect on the polarization spectrum is negligible.

The degree of linear polarization of the fluorescence is related to the angle, α, between the oscillators in absorption and emission by the equation

$$p = \frac{3\cos^2\alpha - 1}{\cos^2\alpha + 3} \qquad (1)$$

In using eq 1 it is assumed that the depolarization is due solely to nonparallel absorbing and emitting oscillators, and not to rotational movements of the fluorescent chromophore in its electronically excited state. As mentioned above the po-

Table I. Fluorescence Polarization and Apparent Angle between the Oscillators in Absorption and Emission of ε-NAD^+ (Calculated Using Equation 1)

	free in glycerol		LDH-ε-NAD^+-oxalate		LADH-ε-NAD^+-pyruvate		GPDH-ε-NAD^+ (1:1)	
λ, nm	p	α, deg	p	α, deg	p	α, deg	p	α, deg
370	0.24	38	0.24	38				
380	0.245	37.5	0.205	40.5	0.235	38	0.21	40
390	0.25	37	0.19	42	0.235	38	0.22	39
400	0.25	37	0.175	43	0.23	38.5	0.23	38.5
410	0.25	37	0.165	43.5	0.23	38.5	0.24	38
420	0.25	37	0.16	44	0.225	39	0.24	38
430	0.25	37	0.155	44	0.215	40	0.235	38
440	0.25	37	0.15	44.5	0.205	40.5	0.23	38.5
450	0.25	37	0.145	45	0.20	41	0.225	39
460	0.25	37	0.14	45	0.19	41.5	0.22	39.5
470	0.25	37	0.13	46	0.18	42.5	0.205	40.5
480	0.25	37	0.12	46.5	0.175	43	0.195	41
490	0.25	37	0.115	47	0.165	43.5	0.18	42.5
500	0.245	37.5	0.11	47	0.155	44	0.165	43.5

larization of fluorescence of ε-NAD^+ in very viscous media justifies this assumption.[10] By means of eq 1 the angles between the oscillators in absorption and emission were calculated for ε-NAD^+ complexes for various emission wavelengths and are presented in Table I. From these values it is clear that, in all the cases studied, the change in direction of the emission oscillator with wavelength is smaller than 10°. The marked dependence of p on α is clarified by considering the rate of change of p with α. By differentiating eq 1 one obtains

$$\frac{dp}{d\alpha} = -\frac{20 \cos \alpha \sin \alpha}{(\cos^2 \alpha + 3)^2} \qquad (2)$$

Thus, $dp/d\alpha = 0$ for $\alpha = 0$ or 90°, and is maximal when $\alpha = 52.9°$. The values obtained for α in the present study (see Table I) are close to the latter value and a strong dependence of polarization on α thus results.

It is interesting to note that the circular polarization of luminescence (CPL) of ε-NAD^+ bound to LADH or to LDH was found to depend only slightly on the emission wavelength.[9] The CPL is determined by the scalar product of the electric and magnetic transition dipole moments involved in the emission, and therefore linearly depends on the cosine of the angle between these two vectors. The changes observed in the direction of the electric transition dipole moment will affect the CPL only slightly if the magnetic transition dipole moment is parallel to it, or nearly so.[21] Thus it appears that the relative orientation of the electric transition dipoles of ε-NAD^+ in absorption and emission is such that small changes in α have a large effect on p, while the same angular changes between the electric and magnetic dipoles in emission have little effect on the CPL due to the relative orientation of these two vectors.

The polarization spectra of the GPDH-ε-NAD^+ complexes also depend on the wavelength of emission (see Figure 2). The fluorescence of ε-NAD^+ bound to GPDH was found to decay biexponentially[16] and thus heterogeneity of emitting species cannot be ruled out. However, as was pointed out before, this heterogeneity cannot explain the variation of p across the emission band, since for a well-behaved fluorophore the polarization should be the same for the various species. It is therefore concluded that also in the case of GPDH-ε-NAD^+ the partial forbidden character of the transition is the cause for the variation of p across the emission band.

The decay parameters as well as the steady-state emission spectrum of the bound ε-NAD^+ were found to be independent of the degree of saturation of the binding sites by the coenzyme analogue. Since the binding of ε-NAD^+ to GPDH is known to show strong negative cooperativity[10] one would expect that if two different classes of binding sites existed these would be sequentially populated by ε-NAD^+, and hence the fluorescence and decay parameters would change with the increase of binding sites occupation. It seems unlikely, therefore, that the biexponential decay is caused by the existence of two classes of coenzyme binding sites in GPDH. Moreover, the biexponential decay of the fluorescence is already observed in the GPDH(ε-$NAD^+)_1$ complex and thus cannot have its origin in two different classes of binding sites. The two emitting species (deduced from the fluorescence decay mechanism) may represent different conformations of bound ε-NAD molecules or may originate in differences in interactions of the ethenoadenine ring with amino acid residues in its binding site. The changes of p with wavelength cannot be due to the existence of the two emitting species also because in that case one would expect p to change monotonously with wavelength while our results clearly show that p has a more complex shape with a maximum at about 420 nm. It may therefore be concluded that each of the two species in the molecular population has a wavelength-dependent polarization spectrum.

The dependence of the fluorescence polarization on the degree of occupation of GPDH binding sites by ε-NAD^+ deserves some further discussion. One possible explanation for the partial depolarization which is observed upon increasing the number of bound ε-NAD^+ molecules is by energy transfer by the Förster mechanism from an originally excited ε-NAD^+ to a second coenzyme molecule bound to the same enzyme molecule. Since GPDH is a symmetric tetramer composed of four identical subunits[18] an efficient energy transfer should result in very drastic depolarization of fluorescence, and in the complex E(ε-$NAD^+)_4$ it should result in complete depolarization. The relatively small depolarization observed for the latter complex with respect to GPDH(ε-$NAD^+)_1$ indicates that efficient energy transfer does not occur. If the depolarization were due to weak energy transfer then the effect would be additive and the polarization should decrease by almost the same amount between E(ε-$NAD^+)_3$ and E(ε-$NAD^+)_4$ as between E(ε-$NAD^+)_1$ and E(ε-$NAD^+)_2$. This clearly does not happen (see Figure 2). It may therefore be concluded that the partial depolarization of ε-NAD^+ fluorescence is due to changes in the environment of bound coenzyme among the different complexes. These changes may reflect either different classes of binding sites (different enough to influence the polarization but not the fluorescence decay parameters) or sequential changes in enzyme conformation which accompany

the binding of increasing number of ϵ-NAD^+ molecules. The latter mechanism has been previously proposed to explain CPL results of ϵ-NAD^+ which, like those of linear polarization presented here, change predominantly upon addition of the second coenzyme molecule.

The results obtained in the present study may be of a more general interest, since variation of p across single electronic bands may be more common than suspected. It was found before for the dansyl chromophore when bound to anti-dansyl antibodies.[19] Several more cases were listed elsewhere.[8] These results lead to some important conclusions. In the first place, whenever p varies across the emission band it is meaningless to present the excitation polarization spectrum without stating precisely what part of the emission band was used in the measurement. The variation of p in the fluorescence spectrum may explain in part why the limiting value of p expected to be 0.5 at the long-wavelength edge of the absorption spectrum is not often attained,[8] since it is common practice to collect a large part of the emission band in polarization studies. The sensitivity of p to the environment of the emitting chromophore in cases involving weak transitions dictates special precaution when p is used to probe the viscosity of the medium in which the chromophore is embedded, e.g., in membranes. If the transition is weak, changes in p may occur due to perturbation of the electronic levels rather than to changes in viscosity. Study of the polarization across the emission band may help verify the eligibility of a fluorophore for such purposes.

Finally it should be noted that fluorophores whose emission is due to a weak transition may be exploited to serve a useful purpose. The sensitivity of their polarization spectrum across the emission band to the environment of the fluorophore may be used as an additional property by which the environment is probed. Such an application of the emission polarization spectrum has been illustrated in the study of the heterogeneity of anti-dansyl anti-bodies.[19]

References and Notes

(1) J. A. Secrist, III, J. R. Barrio, and N. J. Leonard, *Science*, **175**, 646 (1972).
(2) J. R. Barrio, J. A. Secrist, III, and N. J. Leonard, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2039 (1972).
(3) J. R. Barrio, J. A. Secrist, III, and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, **46**, 597 (1972).
(4) J. A. Secrist, III, J. R. Barrio, N. J. Leonard, C. Villar-Palasi, and A. G. Gilman, *Science*, **177**, 279 (1972).
(5) J. A. Secrist, III, J. R. Barrio, N. J. Leonard, and G. Weber, *Biochemistry*, **11**, 3499 (1972).
(6) A. C. Albrecht, *J. Chem. Phys.*, **33**, 156 (1960).
(7) A. C. Albrecht, *J. Mol. Spectrosc.*, **6**, 84 (1961).
(8) I. Z. Steinberg in "Concepts in Biochemical Fluorescence", R. F. Chen and H. Edelhoch, Ed., Marcel Dekker, Vol. 1, New York, N.Y., 1975, p 79.
(9) J. Schlessinger, I. Z. Steinberg, and A. Levitzki, *J. Mol. Biol.*, **91**, 523 (1975).
(10) J. Schlessinger and A. Levitzki, *J. Mol. Biol.*, **82**, 547 (1974).
(11) H. Sund and H. Theorell in "The Enzymes", Vol. 7, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, New York, N.Y., 1963, p 26.
(12) S. F. Velick, *J. Biol. Chem.*, **233**, 1455 (1958).
(13) B. Ehrenberg, Ph.D. Thesis, The Weizmann Institute of Science, 1976.
(14) I. Z. Steinberg and A. Gafni, *Rev. Sci. Instrum.*, **43**, 409 (1972).
(15) G. Weber and B. Bablouzian, *J. Biol. Chem.*, **241**, 2558 (1966).
(16) A. Gafni in "Pyridine Nucleotide Dependent Dehydrogenases", Vol. 2, H. Sund, Ed., Walter de Gruyter, Berlin, 1977, p 237.
(17) P. L. Luisi, A. Baici, F. J. Bonner, and A. A. Aboderin, *Biochemistry*, **14**, 362 (1975).
(18) J. I. Harris and M. Waters in "The Enzymes", Vol. 13, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1976, p 1.
(19) J. Schlessinger, I. Z. Steinberg, and I. Pecht, *J. Mol. Biol.*, **87**, 725 (1974).
(20) K. Mihashi and P. Wahl, *FEBS Lett.*, **52**, 8 (1975).
(21) More generally the dependence of the CPL on direction of the electric transition dipole moment will be minimal when the projection of the magnetic transition dipole moment on the plane defined by the rotating electric vector is parallel to the latter vector. Thus, the CPL may be unaffected by changes in the direction of the electric vector like those found in the present study (see Table I) even if the angle between the magnetic and electric vectors is large, as long as the general requirement stated above is fulfilled.

Electrochemistry of Vitamin B-12. 4. Kinetics and Mechanisms in B-12a–B-12r Oxido-Reduction

N. R. de Tacconi,[1a,b] D. Lexa,[1c] and J. M. Savéant*[1a]

Contribution from the Laboratoire d'Electrochimie de l'Université de Paris VII, 75 221 Paris Cedex 05, France, and the Laboratoire de Biophysique du Muséum d'Histoire Naturelle, 75 005 Paris, France.

Received February 28, 1978

Abstract: Cyclic voltammetry and rotating disk electrode voltammetry on gold, vitreous carbon, and mercury electrodes have been used to investigate the mechanism and the kinetic characteristics of B-12a–B-12r oxido-reduction. Base-on aquo-B-12a and base-on B-12r are the electrochemically reacting species in a large part of the pH range (pH 3–8), giving rise to a slow charge transfer. The charge transfer process involving the base-off forms is markedly more rapid. It begins to compete with the process involving the base-on forms only in very acidic media: below pH 1.5 for B-12r oxidation and below pH 0 for the reduction of B-12a. The nucleotide side-chain opening and closing rate constants have been derived from the evaluation of this competition. Above pH 8 two competing reduction processes are observed: reduction of the hydroxo form through its prior conversion into the aquo form and direct reduction of the hydroxo form. The latter, which completely predominates at pH 12, occurs at a potential negative to the B-12r–B-12s couple, giving rise to an apparent disproportionation phenomenon. The rate of proton abstraction from the aquo-B-12a yielding hydroxo-B-12a has been determined. A general picture summarizing the reaction pathways in the B-12a–B-12r–B-12s system and their thermodynamic and kinetic characteristics is given.

Decreasing from 3 to 1 the oxidation state of the cobalt atom in aquocobalamin, i.e., passing from B-12a to B-12r and B-12s, results in various changes in axial ligandations which are also under the dependence of the acidity of the medium. The equilibrium thermodynamics of these various reactions have been investigated in detail in the second paper of this series.[2] A quantitative description of the stability domains of the three oxidation states of cobalt in aquocobalamin as a function of potential and pH is thus available, providing the values of the characteristic standard potentials, pK_as, and equilibrium constants.

Regarding the B-12r–B-12s couple a kinetic investigation using cyclic voltammetry has provided a description of the oxido-reduction mechanisms at various pHs showing in par-

0002-7863/79/1501-0467$01.00/0