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Dynamic and static quenching of 1,N^6-ethenoadenine fluorescence in nicotinamide 1,N^6-ethenoadenine dinucleotide and in 1,N^6-etheno-9-[3-(indol-3-yl)propyl]adenine

(1,N^6-ethenoadenine derivatives/fluorescence efficiency, emission, and lifetime/fluorescent coenzyme/ϵNAD^+/stacked conformations)

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ABSTRACT **For nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵNAD^+), the fluorescent analog of NAD^+, in neutral aqueous solution the quantum yield has been determined to be 0.028 and the fluorescent lifetime, 2.1 nsec. Simultaneous determination of quantum yields and lifetimes of ϵNAD^+ and of the "half molecule" ϵAMP allows the calculation of the percentage of stacked and open conformations of the dinucleotide. At 25° in neutral aqueous solution there is 45 ± 5% of stacked forms. The value of the fluorescent lifetime observed for ϵNAD^+ is sensitive to fluorescent impurities, especially those containing the ϵ-adenosine moiety, and a purification procedure using high performance liquid chromatography was devised to obtain fluorescently homogeneous preparations.**

In order to study the effect on ϵ-adenosine fluorescence caused by the possible close proximity of a tryptophan in a polypeptide chain or protein, we have prepared 1,N^6-etheno-9-[3-(indol-3-yl)propyl]adenine (ϵAde^9-C_3-Ind^3), a model compound in which indole is used as a neutral substitute for tryptophan. Fluorescence studies on ϵAde^9-C_3-Ind^3 show that the formation of an intramolecular complex results in complete quenching of the ϵ-adenine fluorescence. It is therefore predictable that positioning of the ϵ-adenosine of any fluorescent coenzyme moiety (e.g., ϵATP, ϵADP) in close proximity to a tryptophan in a protein will result in complete fluorescence quenching of the former.

Accumulated evidence on the conformation of nicotinamide adenine dinucleotide, NAD^+, and the reduced form, NADH, in neutral aqueous solution is consistent with a population of open forms, in which the ribose-diphosphate-ribose backbone is extended, and closed forms, in which the heterocyclic rings are "stacked" in close proximity (1–14). Energy transfer from adenine to dihydronicotinamide in NADH in very dilute solution is compatible only with a structure in which the two heterocyclic rings are in close proximity (15, 16). For this case, an energy transfer efficiency of 40% does not allow a determination of the relative amounts of open and stacked forms. Thermodynamic studies by means of ^1H nuclear magnetic resonance NMR on NAD^+ in more concentrated solution gave amounts of folding of 15–40% (2, 8). While a heterogeneity of conformations exists in solution, the coenzyme assumes an open or extended form on binding to dehydrogenases (17 and refs. therein). As part of our work on fluorescently modified nucleotides, dinucleotides, and coenzymes (18–24), we have determined accurately the fluorescence lifetime and the quantum yield of nicotinamide 1,N^6-ethenenoadenine dinucleotide (ϵNAD^+), the fluorescent analog of NAD^+ obtained by treatment of NAD^+ with chloroacetaldehyde. Simultaneous measurement of these quantities and comparison with those for the "half molecule" ϵAMP allow calculation of the equilibrium constant between open and folded conformations of dinucleotide in neutral aqueous solution. We have also prepared ϵNADH and compared its fluorescence lifetime with that of NADH.

These studies relate to intramolecular stacking interactions within the ϵNAD^+ molecule and set a probable upper limit for the interactions within the NAD^+ molecule itself. It is also important to delineate possible interactions between the adenosine moiety of various coenzymes and the individual amino acids, e.g., tryptophan, of various proteins. To do so on a simplified level we synthesized 1,N^6-etheno-9-[3-(indol-3-yl)propyl]adenine (ϵAde^9-C_3-Ind^3) by reaction of 9-[3-(indol-3-yl)propyl]adenine (Ade^9-C_3-Ind^3) (25, 26) with chloroacetaldehyde, thus providing a model in which indole is used as a neutral substitute for tryptophan. From simultaneous measurement of the fluorescence lifetime and the quantum yield of ϵAde^9-C_3-Ind^3 relative to those of the "half molecules" 1,N^6-etheno-9-propyladenine (ϵAde^9-C_3) and 3-propylindole (Ind^3-C_3), we are able to predict the fluorescence quenching effect of bringing the ϵ-adenine moiety into close proximity with the indole of tryptophan.

MATERIALS AND METHODS

ϵNAD^+: Preparation, Purification, and Stability. Since ϵNAD^+ is susceptible to hydrolysis of the pyrophosphate bond and since the quantum yield of ϵAMP is approximately 20 times greater than that of ϵNAD^+, careful purification is required. The modified coenzyme ϵNAD^+ was prepared according to the method of Barrio *et al.* (20), followed by purification by high performance liquid chromatography using an Aminex A-28 anion exchange resin equilibrated with 0.05 M formic acid and eluted with the same solvent. The column was operated at approximately 400 lb./inch^2 (27.6 MPa). The eluate was monitored by absorbance at 254 nm, recorded on a Hewlett-Packard recorder modified as described previously (27). As a criterion of purity we used identical lifetimes determined by both phase shift and modulation (28, 29). This technique is very sensitive to the presence of more than a single fluorescent decay, and the presence of ϵAMP in our preparation would be expected to

Abbreviations: In the abbreviation ϵNAD^+ for nicotinamide 1,N^6-ethenoadenine dinucleotide, ϵ stands for the etheno bridge and is also suggestive of the molar absorbance term and of the fluorescence emission. The abbreviation ϵAde^9-C_3-Ind^3 for 1,N^6-etheno-9-[3-(indol-3-yl)propyl]adenine contains the approved IUPAC-IUB symbols [*Biochemistry*, 9, 4022 (1970)] and proposed linkage symbols [Cohn, W. E., Leonard, N. J. & Wang, S. Y., (1974) *Photochem. Photobiol.* 19, 89].

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lengthen the observed lifetime and give heterogeneous lifetimes differing by 0.5 to 1 nsec by the two methods. Our lifetimes obtained by the two methods were within 0.2 nsec, and were determined within several days of purification. Storage for longer periods of time in frozen solution resulted in the observation of heterogeneous lifetimes.

εNADH: Preparation and Purification. Highly purified εNAD$^+$ (above) was incubated with horse liver alcohol dehydrogenase (EC 1.1.1.2; alcohol:NADP$^+$ oxidoreductase) in 0.1 M Tris·HCl, pH 8.5 containing 3% ethanol for 1–2 hr. The crude reaction mixture was applied to an Aminex A-28 anion exchange column and eluted with 0.3 M ammonium chloride containing 25% (v/v) ethanol.

1,N^6-Etheno-9-[3-(indol-3-yl)propyl]adenine (εAde^9-C_3-Ind^3). A 200-mg sample of 9-[3-(indol-3-yl)propyl]adenine (25) was stirred at 25° in 2 M aqueous chloroacetaldehyde with ethanol added for solubility. When the reaction had proceeded to completion, as shown by thin layer chromatography (Eastman Chromatogram sheets) on silica gel with fluorescent indicator in the solvent system: chloroform–methanol–acetic acid (90:5:5), the solution was decolorized with charcoal and the solvent was evaporated. Recrystallization from ethanol–water provided an analytical sample, mp 137–139°. (*Analysis:* Calculated for $C_{18}H_{16}N_6 \cdot \frac{1}{2}H_2O$: C, 66.45; H, 5.27; N, 25.83. Found: C, 66.13; H, 5.25; N, 26.10.)

Relative Quantum Yields were determined on a Hitachi Perkin-Elmer MPF-2A spectrofluorometer. Relative quantum yields for εNAD$^+$ compared with εAMP were determined in 0.05 M phosphate, pH 7, by comparing the area under the emission curve for a solution containing εNAD$^+$ before and after hydrolysis with snake venom phosphodiesterase when the samples were excited at 305 nm. The final relative quantum yields were obtained after correction for the hypochromism of the εNAD$^+$. The quantum yield of εNAD$^+$ was obtained by comparison with quinine sulfate, which has a quantum yield of 0.70 in 0.5 M H_2SO_4 (30). Similarly, solutions containing equal absorbance of 1, N^6-etheno-9-[3-(indol-3-yl)propyl]adenine and of 1,N^6-etheno-9-propyladenine (31) plus 3-propylindole (25) were compared when excited at 320 nm. At this wavelength, the absorption of indole is negligible.

Fluorescence Lifetimes were determined with the cross-correlation fluorometer described previously (28, 29), using a light modulating frequency of 14.7 MHz. The exciting light was selected by a monochromator and filtered through a Corning CS-7-54 filter. The emitted light was filtered through a Corning 0-54 or 0-51 filter. Fluorescence lifetimes greater than 2 but less than 20 nsec were identical by phase and modulation to within 0.2 nsec. Above 20 nsec the lifetimes determined by phase and modulation were homogeneous within 0.5 nsec.

RESULTS AND DISCUSSION

In the fluorescent and biologically active analog of NAD$^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide (εNAD$^+$) (21, 22), the ε-adenosine fluorescence is quenched intramolecularly by nicotinamide. If the diphosphate is hydrolyzed, less quenching is observed (19, 32, 33), the same behavior having been observed with other fluorescent coenzyme analogs (22, 34). Using our highly purified εNAD$^+$ preparation, we have now determined the fluorescence properties of the dinucleotide analog. *The fluorescence lifetime of εNAD$^+$ in neutral aqueous solution at 25° is 2.1 nsec and the quantum yield, compared with 0.70 for quinine sulfate (30), is 0.028.* The lifetime of εAMP reported previously (19) is 23.0 nsec and the quantum yield is 0.56 under the same conditions. It is important to use highly purified εNAD$^+$ for fluorescent lifetime and quantum yield determinations. The coenzyme NAD$^+$ is known to undergo hydrolysis to AMP, ADP, and ADP ribose (35). Since these products in the case of εNAD$^+$ degradation would be approximately 20 times more fluorescent than εNAD$^+$ itself, a 0.1% chemical impurity would be equivalent to a 2% fluorescent impurity. Also, the longer fluorescence lifetime of the ε-adenosine moieties of the hydrolytic products would lengthen the εNAD$^+$ lifetime observed. Such impurities are probably responsible for the longer lifetime quoted elsewhere (36).

While the fluorescence lifetime of εNAD$^+$ is 9.2% of the value for εAMP, the relative quantum yield is only 5% of that observed after snake venom treatment, taking into account a correction for hypochromism in the dinucleotide, an observation which is also consistent with a folded structure (22, 37–42).

We have shown previously (23) that ε-adenosine can form dark complexes with intramolecularly connected nucleic acid bases. Formation of such a complex decreases the quantum yield but does not affect the fluorescence lifetime. This process is static quenching. Alternatively, the fluorophore can undergo intramolecular collisional quenching, dynamic quenching, which is competitive with emission and affects the fluorescence lifetime. In a molecule such as εNAD$^+$ that may exist as an equilibrating mixture of both stacked and open forms, both static and dynamic quenching can contribute to the lowered quantum yield, and simultaneous measurement of relative quantum yield and fluorescence lifetime allows calculation of the equilibrium constant (21, 23, 25, 26). Assuming that the quantum yield of internally com-

Table 1. Temperature dependence of fluorescence lifetime and quantum yield and intramolecular complexing of εNAD$^+$

Temperature (°C)	Fluorescence lifetime[a]		Relative quantum efficiency[b]	Fluorescence efficiency		Degree of internal association $(1-\alpha)$ (%)[c]	Equilibrium constant, K_a $(1-\alpha)/\alpha$
	εAMP (nsec)	εNAD$^+$ (nsec)		Static γ	Dynamic τ/τ_0		
5	26.8	3.4	0.056	0.44	0.13	56	1.3
15	25.3	2.7	0.054	0.51	0.11	49	0.96
25	23.0	2.1	0.050	0.55	0.091	45	0.82
30	22.0	1.9	0.049	0.57	0.086	43	0.75
40	20.2	1.5	0.047	0.63	0.074	37	0.59

[a] Fluorescence lifetimes for both εAMP and εNAD$^+$ were determined under the same conditions. Excitation was at 305 nm.
[b] Relative quantum efficiencies were determined by integration of fluorescence emission spectra before and after complete enzymatic hydrolysis, and spectra were corrected for differences in absorption. Excitation at 305 nm.
[c] Error in $(1-\alpha)$ is ±5%.

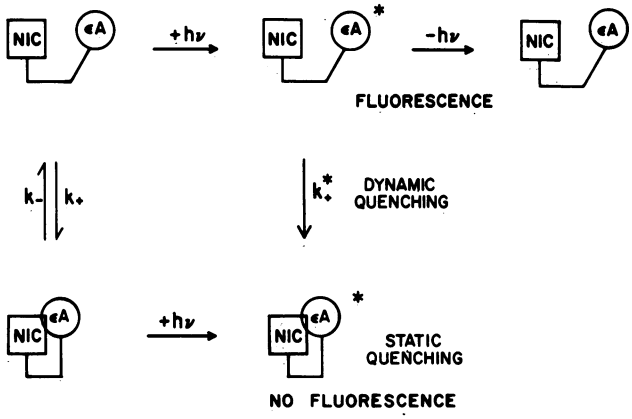

FIG. 1. Kinetic scheme for the intramolecular quenching of ϵNAD^+ in neutral aqueous solution.

plexed forms is negligible compared with open forms, the parameter $\gamma = (F/F_0)\cdot(\tau_0/\tau)$ may be introduced (29), where F_0 and τ_0, F and τ represent the quantum efficiency and fluorescence lifetime in the absence and presence, respectively, of intramolecularly connected nicotinamide. Thus, γ corresponds to the fraction of absorption transitions by free, unquenched fluorophore in the ground state relative to the total number of absorptions. Values for γ as a function of temperature are reported in Table 1. In the case where the absorbance of ϵNAD^+ and of ϵAMP plus NMN are equal, as we have adjusted it in our experiments, γ is equal to α, where α is the fraction of molecules in open conformations and $1 - \alpha$ (Table 1) is the fraction of stacked forms. Under these conditions ϵNAD^+ is 45 ± 5% stacked. The equilibrium constant for stacking, K_a, equals $(1 - \alpha)/\alpha$.

The kinetic scheme for fluorescence quenching is shown in Fig. 1. The rate constant in Fig. 1 for dynamic quenching, $k_+^* = (1/\tau) - (1/\tau_0)$, is 4.32×10^8 sec^{-1} at 25°. From a van't Hoff plot of ln K_a against temperature, values of the thermodynamic constants were calculated. Both enthalpy and entropy values are negative, $\Delta H^0 = -3.6 \pm 0.2$ kcal/mol and $\Delta S^0 = -12.5 \pm 0.7$ e.u. These figures may be compared with $\Delta H^0 = -4.3$ kcal/mol and $\Delta S^0 = -11.3$ *e.u.* for the same equilibrium in FAD (43).

Using a high performance liquid chromatography system we have purified $\epsilon NADH$. When the reduced analog was excited at wavelengths ≥360 nm, the fluorescence lifetime observed for $\epsilon NADH$, 0.40 ± 0.03, is identical with that for NADH (30), both representing the lifetime of dihydronicotinamide fluorescence. The ε-adenosine can be excited selectively in aqueous solution; however, due to energy transfer in the stacked forms, the emission is from both fluorophores. In propylene glycol, a solvent in which the dinucleotide NADH has been shown to be in the open form (30), excitation of $\epsilon NADH$ at 265 nm, where most of the absorption is due to the ε-adenosine, results in an emission maximum at 410 nm characteristic of the same moiety.

Intermolecular stacking interactions between tryptophan or related indolic compounds and the nucleic acid bases have been demonstrated, *inter alia*, by reflectance and luminescence studies in aggregates formed in frozen aqueous solutions (44–46). Intramolecular interaction between indole and the nucleic acid bases adenine, guanine, thymine, and cytosine connected by a trimethylene bridge has been shown to cause complete quenching of the indole fluorescence in dilute aqueous solution at 25° (25, 26). Another method of assessing such intramolecular interactions is to examine the specific case of the quenching of ε-adenine fluorescence in another model containing the trimethylene bridge, namely 1,N^6-etheno-9-[3-(indol-3-yl)propyl]adenine (ϵAde^9-C_3-Ind^3). This is made by the reaction of Ade^9-C_3-Ind^3 with chloroacetaldehyde, which incorporates the 1,N^6-etheno bridge in the adenine moiety. The fluorescence lifetime of ϵAde^9-C_3-Ind^3 is 1.3 nsec (determined by excitation at 320 nm where absorbance of indole is negligible) and the relative quantum yield is 0.022. Using 23.8 nsec as the fluorescence lifetime of ϵAde^9-C_3 (23), the parameter $\gamma = 0.40$ is consistent with a population of conformations in which intramolecularly complexed forms (60% ± 10%) cause complete quenching of ε-adenine fluorescence. From this model compound we predict that positioning of ε-adenosine in close proximity to the indole of tryptophan in a polypeptide chain or protein will result in complete quenching of ε-adenosine fluorescence.

It is interesting to compare the amount of intramolecular complexation in ϵAde^9-C_3-Ind^3 (60% ± 10%) with that in Ade^9-C_3-Ind^3 (53 ± 10%) (25, 26). In that connection, from a plot of ln K_a against temperature for Ade^9-C_3-Ind^3, $\Delta H^0 = -8.1 \pm 0.9$ kcal/mol and $\Delta S^0 = -26.6 \pm 3.0$ e.u. Equivalent or greater complexation is observed in other systems in which ε-adenosine replaced adenosine (23, 31, 47). Where the difference is real, the increased stacking is probably due to increased van der Waals-London dispersion forces dependent upon aromatic ring cross section. Similarly, then, in the case of the stacking interaction reported here for dilute aqueous solution, the 45 ± 5% value for stacking in ϵNAD^+ represents an upper limit to the amount of stacking in NAD^+ itself.

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