Flavin 1, N⁶-Ethenoadenine Dinucleotide: Dynamic and Static Quenching of Fluorescence

(FAD/1,N⁶-ethenoadenine derivative/fluorescence emission/coenzyme analogue/energy transfer)

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ABSTRACT Flavin 1,N⁶-ethenoadenine dinucleotide (eFAD) was prepared by the action of chloroacetaldehyde on flavin adenine dinucleotide. This compound, which has two potential fluorophores, e-adenine and isalloxazine, shows extremely efficient energy transfer from the former to the latter. The fluorescences of both moieties are greatly diminished in the intact molecule. Determination of the fluorescence yields and lifetimes leads to the conclusion that at 20° in neutral aqueous solution eFAD exists mainly (90%) as an internally complexed or stacked form.

We have obtained the fluorescent derivative of flavin adenine dinucleotide (FAD), flavin 1,N⁶-ethenoadenine dinucleotide (eFAD) (1) in pure form by extensive chromatography, monitored by means of the homogeneity of the fluorescence lifetimes. In this modified coenzyme, prepared by the action of chloroacetaldehyde on FAD, there are two potential fluorescent groups, the 1,N⁶-ethenoadenine (eA or eAde) moiety (1) and the isalloxazine moiety. Their quenching and energy-transfer properties are of special interest (2,t), and the fluorescence yields and lifetimes provide the basis for assigning the relative proportions of open and stacked or folded conformations (3).

MATERIALS AND METHODS

Flavin 1,N⁶-Ethenoadenine Dinucleotide. Reaction of FAD with a 50:fold excess of 1.5–2.0 M chloroacetaldehyde (pH 4.5) for 72 hr at 23° with protection from light produced the 1,N⁶-etheno-bridged FAD. The extent of reaction was monitored by use of polyethyleneimine thin-layer chromatograms (Polygram, Brinkman Instruments) which were developed with 1.0 M LiCl or isobutyric acid: NH₄OH:H₂O 75:1:24, v/v. eFAD was extensively purified by column chromatography on DEAE-Sephadex, eluting with either 100 mM phosphate buffer (pH 6.8) or a gradient of ammonium formate (pH 4.0), 0.025–1.0 M, following the excellent method of purification of FAD supplied by Massey and Swoboda (4). Homogeneity of the fluorescence lifetimes, as determined by phase and modulation by use of the cross-correlation fluorometer (5), was found to be the most sensitive criterion of purity and was therefore extremely useful for following the purification procedure. All purification steps were performed at 5° and in the dark.

Hydrolysis of eFAD by Phosphodiesterase I from Crotalus adamanteus venom (EC 3.1.4.1) (Sigma). The assay was performed at 23° in 3-ml reaction mixtures containing 100 mM phosphate buffer (pH 7.0) and eFAD (0.2 OD at 450 nm) by following the increase in fluorescence intensity of the e-adenine moiety (410 nm) when phosphodiesterase I was added in a quantity sufficient to produce complete hydrolysis in 30–40 min.

![Fluorescence emission spectrum of eFAD in 0.1 M aqueous phosphate buffer at pH 7.0.](image-url)
RESULTS AND DISCUSSION

In FAD, the energy transfer from the 1,N⁴-ethenoadenine moiety to the isoalloxazine moiety is nearly 100% efficient. Excitation at 305 nm, where most of the absorption is due to the eAde, and at 405 nm, where the flavin absorbs exclusively, yield identical lifetimes and quantum efficiencies for the isoalloxazine fluorescence at 525 nm. Dramatic proof of the quenching of the eAde moiety in FAD is shown by enzymatic hydrolysis. A comparison of the fluorescence emission spectra before and after the hydrolysis of the FAD with snake venom phosphodiesterase I (Figs. 1 and 2) clearly shows a large increase in fluorescence emission at 410 nm when the coenzyme analog is cleaved into the two parts. The emission of the coenzyme analog at 410 nm (Fig. 1) is negligible (1/2000) when compared with the fluorescence efficiency at 410 nm after complete enzymatic hydrolysis. From a similar comparison of efficiencies at 525 nm, it is evident that the isoalloxazine moiety is also significantly quenched in the eFAD.

The lifetimes and relative quantum efficiencies for the isoalloxazine fluorescence in FMN, FAD, and eFAD upon excitation at 450 nm are shown in Table 1 and were used to determine the dynamic and static quenching parameters (Table 2) in the kinetic scheme of Fig. 3 [see also ref. (8)]. At 20° in neutral aqueous solution eFAD exists mainly (90%) as an internally complexed or stacked form. The intramolecular interactions between the e-adenine and flavin moieties are therefore stronger than those in the normal coenzyme FAD, where 82% is stacked. The 2-fold increase in the equilibrium constant of eFAD must be due to the additional van der Waals–London dispersion forces provided by addition of a third ring to the adenine moiety. By contrast, the rate constants for the dynamic quenching of eFAD and FAD, solely a diffusion phenomenon, are, as expected, nearly identical. The observations that resonance energy transfer from the modified adenine to the isoalloxazine moieties is close to 100% efficient is reasonable because of the long fluorescence lifetime of the eAde (23 nsec) (1) and the restricted separation of the two moieties in the dinucleotide.

† Even in the extended form of eFAD, the maximum distance between moieties is about 20 Å.
Considering the high degree of freedom of the dinucleotide and the high rate of dynamic quenching (1.7 \times 10^{9} \text{sec}^{-1}) or diffusion of the ε-adenine and the isalloxazine portions toward each other, energy transfer would be expected to occur with the high probability observed (6–8).

In terms of coenzyme activity, Harvey and Damle (2) have already pointed out that εFAD provided almost full enzymatic activity with apoglucose oxidase, but no activity with apo-D-amino acid oxidase. Determination of the activity of εFAD in comparison with FAD, together with application of the present data concerning fluorescence and conformation (ref. 3 and this work), should provide additional information regarding enzyme mechanisms and binding modes.

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