A FLUORESCENT MODIFICATION OF FLAVIN ADENINE DINUCLEOTIDE

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Received 14 July 1972

1. Introduction

Recent studies have shown that the reaction of chloroacetaldehyde with adenine or cytosine derivatives leads to the formation of highly fluorescent compounds [1–4]. The present paper describes the condensation of chloroacetaldehyde with flavin adenine dinucleotide (FAD) resulting in a biologically active 1,N^6-ethenoadenosine analog (abbreviated εFAD, fig. 1).

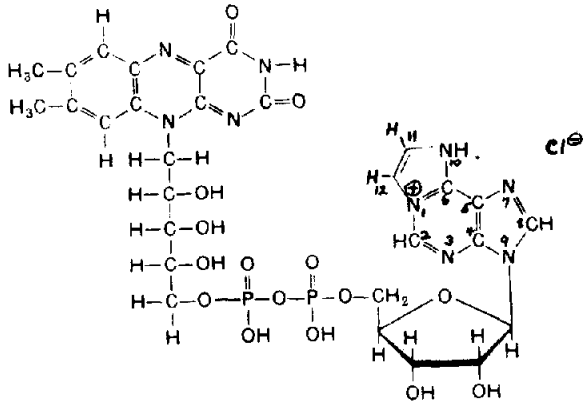

Fig. 1. εFAD.

2. Materials and methods

Redistilled chloroacetaldehyde diethyl acetal (Aldrich Chemical Co., Milwaukee, Wisc.) was hydrolyzed by refluxing with 5% H_2SO_4 for 1 hr. The chloroacetaldehyde obtained by distillation (b.p. 85–6°; semicarbazone, m.p. 148° [5]) was dissolved in water to yield an approx. 2 M solution. This stock solution (unadjusted pH approx. 4.0) when kept at 2° was stable for at least 1 month.

εFAD·HCl (flavin-1, N^6-ethenoadenine dinucleotide hydrochloride): A solution of 83 mg (0.1 mmoles) of flavin-adenine dinucleotide (disodium salt, 96% purity) in 20 ml of 2 M aqueous chloroacetaldehyde was stirred at 37° for 24 hr and then evaporated to dryness on a flash evaporator at a temperature $< 40°$. Recrystallization of the residue from H_2O–EtOH–Et_2O furnished 80 mg (88% yield) of orange-yellow crystals, m.p. $> 300°$. The sample was lyophilized from deuterium oxide to remove exchangeable protons and then dissolved in D_2O. The NMR spectrum (obtained using a Varian T-60 spectrometer with silapentanesulfonate as internal standard) exhibited a pair of doublets at δ8.18 and 8.45 corresponding to the 11- and 12-H's of the 1,N^6-ethenoadenine portion.

Glucose oxidase apoprotein was prepared according to the acid ammonium sulfate method of Swoboda [6]. The apoprotein of D-amino acid oxidase was prepared by the KBr dialysis method [7]. All nucleotides and enzymes were obtained from Sigma Chemical Co., St.Louis, Mo.

Absorption spectra were recorded on a Bausch and Lomb 505 spectrophotometer; uncorrected fluorescence spectra were obtained using a recording spectrofluorometer [8] equipped with a 450 W xenon lamp.

3. Results and discussion

The absorption spectrum of εFAD is virtually identical to that of FAD from 340 nm to 550 nm (fig. 2). However, εFAD shows a stronger absorption in the region from 270 nm to 330 nm and exhibits

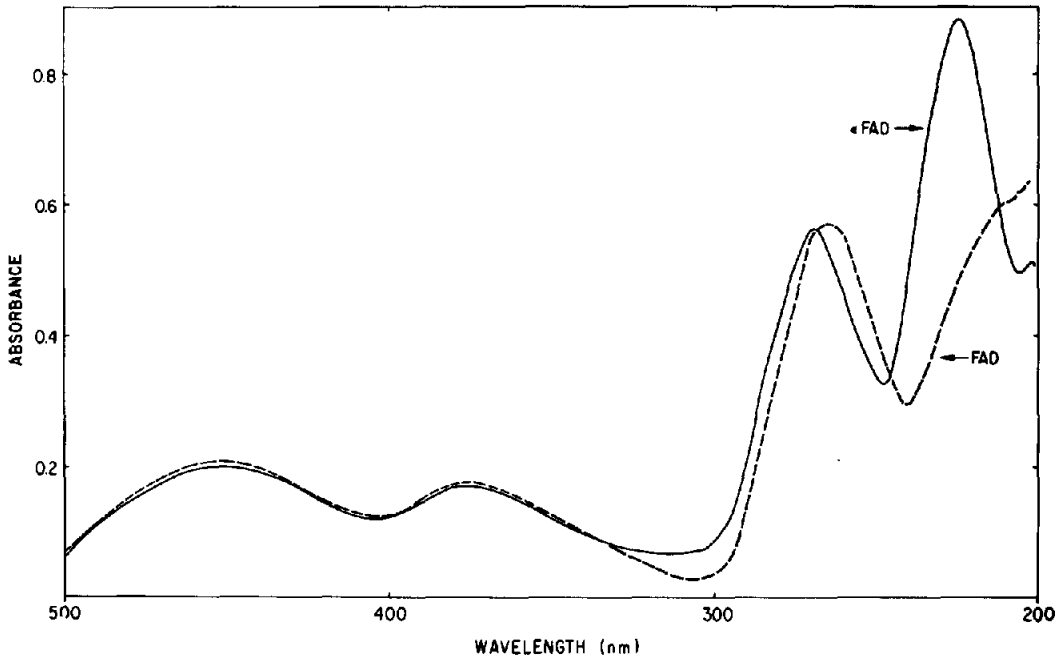

Fig. 2. Absorption spectra of FAD (18 μM) or εFAD (18 μM) in 10 mM potassium phosphate buffer, pH 7.0.

a well-defined peak at 225 nm which is not present in the spectrum of FAD. The spectral differences in the ultraviolet region are consistent with the modification of the purine moiety of FAD since ε-adenosine, in contrast to adenosine, exhibits an intense absorption peak at 230 nm, weaker absorption peaks at 275 nm and 265 nm, and a broad shoulder from 280 nm to 340 nm [2].

εFAD contains two fluorescent moieties, the ε-adenosine and the flavin component. Through a judicious selection of excitation and emission wavelengths, it is possible to selectively observe the fluorescence properties of either the ε-adenosine or the flavin portion of the coenzyme. For example, excitation of εFAD at 300 nm, the excitation maximum for ε-adenosine, results in an emission peak at 410 nm (characteristic of ε-adenosine derivatives) which is absent in the spectrum obtained with FAD (fig. 3). εFAD also exhibits a second emission peak at 525 nm which is substantially greater than seen with an equal concentration of FAD (fig. 3). In contrast, the emission of εFAD and FAD are virtually identical when excited at longer wavelengths where there is no absorption of light by the ε-adenosine portion of the dinucleotide.

The enhanced flavin emission resulting from excitation at 300 nm indicates resonance energy transfer from the ε-adenosine moiety to the flavin part of the coenzyme. The efficiency of this transfer is approx. 50% as calculated from the excitation spectrum of the energy acceptor [9]. The fluorescence of the ε-adenosine and the flavin portions of εFAD are markedly quenched when compared to an equimolar solution of εAMP and FMN. These observations suggest that in aqueous solution the ε-aderine and isoalloxazine rings of εFAD exist in a stacked conformation, similar to that proposed for FAD [10].

εFAD provided almost full enzymic activity with apoglucose oxidase (table 1). This restoration of activity by εFAD was not due to contamination with unreacted FAD since apo-D amino acid oxidase is activated by the FAD but shows no activity in the presence of the εFAD preparation (table 1). While a more extensive investigation of other flavoproteins will be required to fully evaluate the biological activity of εFAD, the present data suggest that the FAD analog may be useful in the study of enzyme mechanisms. For example, the efficiency of intramolecular energy transfer will depend on the

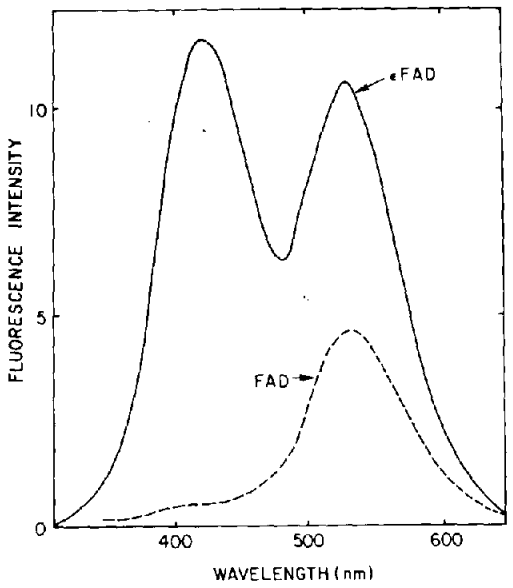

Fig. 3. Fluorescence emission spectra. FAD or εFAD (13 μM) in 5 mM potassium phosphate buffer, pH 7.0, excitation at 300 nm.

relative spatial orientation of the two planar ring systems of εFAD; thus, it may be possible to determine if the enzyme-bound εFAD exists in a stacked or open conformation. Also, reduced εFAD is fluorescent (in contrast to $FADH_2$) and may be potentially useful in investigating interaction between reduced coenzyme and apoprotein.

Acknowledgement

We thank Professor J.M. Cross, School of Pharmacy, Rutgers University, New Brunswick, N.J., for the use of the Varian T-60 NMR Spectrometer. This work was supported in part by NSF grant GB 26081.

Table 1
Coenzyme activity of εFAD.

Conditions	Enzyme activity [a] (ΔA_{340}/min)
D-amino acid oxidase apoprotein	0.06
D-amino acid oxidase apoprotein + FAD [b]	0.45
D-amino acid oxidase apoprotein + εFAD [b]	0.07
Glucose oxidase apoprotein	0.03
Glucose oxidase apoprotein + FAD [b]	0.54
Glucose oxidase apoprotein + εFAD [b]	0.42

a) The D-amino acid oxidase assay contained 0.16 M Tris (pH 8.3), 0.0373 M D-alanine, 0.25 mM NADH, 0.33 μg/ml catalase, 16 μg/ml lactate dehydrogenase, and approx. 10 μg/ml apo-D-amino acid oxidase. Reaction followed by decrease in absorbance at 340 nm. The glucose oxidase assay contained 0.083 M Tris (pH 7.0), 2.7×10^{-4} M o-dianisidine, 0.092 M glucose, 3 μg/ml peroxidase and approx. 8 μg/ml apoglucose oxidase. Reaction followed by increase in absorbance at 346 nm.

b) Apoprotein preincubated 15 min with 4×10^{-7} M FAD or εFAD as indicated.

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