Structural Requirements of the Flavin Moiety of Flavin–Adenine Dinucleotide for Intramolecular Complex Formation*

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ABSTRACT: Fluorescence quenching as a function of pH of solutions of analogs of riboflavin 5′-phosphate (FMN) and of flavin–adenine dinucleotide (FAD) has been examined to characterize the structural requirements for formation of the intramolecular donor–acceptor complex in the dinucleotide. The 3-methyl analog of FMN is nearly as fluorescent as FMN, and the corresponding derivative of FAD can form an intramolecular complex which appears similar to FAD. The 6,7-dichloro analog of FMN is considerably less fluorescent, and its FAD analog does not appear to complex well intramolecularly. Flavin–adenine(1-*N*-oxide) dinucleotide is unable to exist as a stable intramolecular complex. Since replacements of the glycityl chains of FMN analogs with ω-hydroxyalkyl substituents result in some increases of fluorescence, a slight quenching effect may be attributable to secondary hydroxyl groups. The 4′-hydroxybutyl, 5′-hydroxypentyl, and 6′-hydroxyhexyl analogs of FAD form intramolecular complexes with pH stabilities essentially identical with that of FAD. The 3′-hydroxypropyl, DL-glycero, D-erythro, and D-allo analogs of FMN are as fluorescent as FMN, but the corresponding FAD analogs appear to have steric restrictions which prevent formation of intramolecular complexes. As found earlier for FAD, in all cases where intramolecular complexing of FAD analogs can occur neutral to slightly acid conditions allow complexing, whereas strong acid decreases association by protonating both the 6-amino group of adenine and the flavin.

Since most of the flavin in organisms is in the form of flavin–adenine dinucleotide (FAD)[1] which has been found to be the prosthetic group of most flavoproteins (Beinert, 1960), numerous studies have been made on the chemical and physical properties of FAD. As early as 1938, it was observed that the molar extinction coefficient of FAD at 260 mμ is less than the sum of the molar extinction coefficients of its component parts, FMN and AMP (riboflavin 5′-phosphate and adenosine 5′-phosphate) at this wavelength (Warburg and Christian, 1938a,b). Hydrolysis of FAD raises the absorbance at 260 mμ to that expected for the sum of FMN and AMP alone (Whitby, 1953). Studies of fluorescence emission revealed that purines could quench the fluorescence of flavins (Weil-Malherbe, 1946; Burton, 1951), which correlated well with the view that the internal quenching that takes place in FAD reflects an association between the flavin and the adenine moieties (Bessey *et al.*, 1949; Weber, 1950). Quenching of the fluorescence of FMN by purines and pyrimidines as well as internal quenching in adenylate-replaced analogs of FAD has been examined in a recent study (Tsibris *et al.*, 1965) on donor–acceptor complexes relating to the intramolecular association of the riboflavin and adenosine moieties of FAD. However, though a number of compounds that quench the fluorescence of riboflavin were shown to quench similarly the fluorescence of its 3-methyl derivative (Harbury and Foley, 1958), no definitive evidence has been available on the general structural requirements placed on the flavin moiety of FAD in the formation of its internally complexed species.

The present investigation was undertaken to assess the requirements and restrictions pertaining to the structure of the flavin portion of FAD in the formation of its intramolecular donor–acceptor complex. The technique of measuring fluorescence quenching was used to examine the extent of intramolecular association between adenylate and substituted FMN portions of FAD analogs. As a supplement to data available about the role of the adenine moiety in quenching fluorescence, the 1-*N*-oxides of AMP and FAD were also examined.

Experimental Section

Materials. AMP, FMN, and FAD were the purest available from Sigma Chemical Co. 4-Morpholine *N,N′*-dicyclohexylcarboxamidinium adenosine 5′-phosphoromorpholidate was obtained from California Biochemical Corp. The corresponding morpholidate of 5′-AMP 1-*N*-oxide was prepared by reac-

* From the Graduate School of Nutrition and Biochemistry Section of the Division of Biological Sciences, Cornell University, Ithaca, N. Y. *Received August 30, 1965.* This investigation was supported in part by a research grant (AM-04585-05) from the National Institutes of Health and by funds from the State University of New York.

† This work will form part of a dissertation to be presented by B. M. C., National Institutes of Health predoctoral fellow, to the Graduate School of Cornell University in partial fulfillment of the requirements for the Ph.D. degree.

[1] Abbreviations used in this work: FMN, riboflavin 5′-phosphate; FAD, flavin–adenine dinucleotide, AMP, adenosine 5′-phosphate.

tion of the *N*-oxide (Stevens *et al.*, 1959) with dicyclohexylcarbodiimide and morpholine in *t*-butyl alcohol (Moffatt and Khorana, 1961). 3-Methylriboflavin[2] was supplied by the research laboratories of Merck Sharp and Dohme. 6,7-Dichlororiboflavin was synthesized by condensation of *N*-(2-amino-4,5-dichlorophenyl)-1-D-ribamine with alloxan (Kuhn and Weygand, 1935). Other flavins were synthesized by known methods (Chassy *et al.*, 1965). The monophosphate ester of each flavin was prepared by phosphorylation of the terminal hydroxymethyl group with dichlorophosphoric acid (Flexser and Farkas, 1952). The products were purified by chromatography on Whatman No. 3MM paper using *n*-butyl alcohol–acetic acid–water (5:2:3, v:v:v) as the ascending solvent. Elution with water and evaporation to final dryness in anhydrous pyridine yielded the pyridinium flavin phosphates. These salts were allowed to react with the 5′-phosphoromorpholidate of adenosine or adenosine 1-*N*-oxide in equal volumes of anhydrous pyridine and *o*-chlorophenol (Moffatt and Khorana, 1958) or in 5% anhydrous pyridine in anhydrous benzyl alcohol (Chassy and McCormick, 1965). The crude analogs of FAD were purified by chromatography on acid-washed Whatman No. 3MM paper with *n*-butyl alcohol–acetic acid–water (4:1:5, v:v:v; upper phase) as the ascending solvent. The FAD analogs were eluted with 0.01 M potassium phosphate buffer at pH 7.

Methods. Flavins were located in paper chromatograms by illumination under an ultraviolet lamp. After elution, the concentration of flavin solutions was adjusted by measuring absorbance at 450 mμ with a Beckman DU spectrophotometer. Solutions used for determination of the pH profile of the fluorescence emission contained 1 μM flavin in 0.2 M buffers of glycine–HCl, pH 3; sodium acetate, pH 4–5; sodium phosphate, pH 6–7. Though a change in the ionic strength of the medium has a slight effect on formation of a riboflavin–adenosine complex (Tsibris *et al.*, 1965), no significant difference in the magnitude of such an effect could be detected among those flavins tested in any one of the present buffers. Quenching of fluorescence by 5 mM AMP or AMP 1-*N*-oxide was measured with 10 μM solutions of FMN or FMN analog in 0.05 M potassium phosphate buffer at pH 7. Measurements of fluorescence were done at room temperature with an Aminco-Bowman spectrophotofluorometer (No. 4–8106) which was equipped with a xenon lamp (No. 416–992), a photomultiplier tube (1P 21), and slit arrangement No. 2. The activating wavelength was set at an optimum of 445 mμ and fluorescence read at 520 mμ. The absorption and emission spectra of all flavins tested throughout the pH range exhibit maxima near these wavelengths.

Results

The chromatographic behavior of flavins in both slightly acid and alkaline solvents before and after hydrolysis of the FAD analogs is indicated by the data in Table I. The R_F values for the "control" (non-hydrolyzed) samples represent the original dinucleotides, whereas those which were "hydrolyzed" represent the mononucleotides formed during acid hydrolysis. Similar to FAD, all its analogs migrate very slowly in the more acid organic solvent, but considerably faster in the alkaline aqueous solvent. After hydrolysis, the resultant FMN analogs show greater mobilities under both conditions. Those flavins with less polar side chains move somewhat faster in the less polar butanol-containing solvent. These observations helped confirm

TABLE I: Chromatographic Characteristics of FAD Analogs before and after Hydrolysis.

Flavin	R_F Values			
	Solvent 1[a]		Solvent 2[b]	
	Control	Hydrolyzed[c]	Control	Hydrolyzed[c]
FAD	0.03	0.12	0.41	0.63
FA(1-*N*-oxide)D	0.03	0.12	0.38	0.63
3′-Hydroxypropyl-FAD	0.04	0.18	0.52	0.63
4′-Hydroxybutyl-FAD	0.04	0.19	0.50	0.60
5′-Hydroxypentyl-FAD	0.05	0.23	0.47	0.60
6′-Hydroxyhexyl-FAD	0.06	0.28	0.44	0.57
DL-Glycero-FAD	0.03	0.16	0.54	0.64
D-Erythro-FAD	0.04	0.15	0.51	0.63
2′-Deoxyribo-FAD	0.04	0.14	0.44	0.57
D-Allo-FAD	0.03	0.09	0.35	0.57
3-Methylribo-FAD	0.04	0.18	0.35	0.60
6,7-Dichlororibo-FAD	0.04	0.13	0.41	0.63

[a] Solvent 1 was *n*-butyl alcohol–acetic acid–water (4:1:5, upper phase). [b] Solvent 2 was 5% aqueous disodium phosphate. [c] Hydrolysis was effected after 30 min in boiling 1 N HCl.

[2] Trivial names for flavin analogs indicate substituent changes in the structure of riboflavin, 6,7-dimethyl-9-(1′-D-ribityl)isoalloxazine: 3-methylriboflavin, 3,6,7-trimethyl-9-(1′-D-ribityl)-isoalloxazine; 6,7-dichlororiboflavin, 6,7-dichloro-9-(1′-D-ribityl)isoalloxazine; 3′-hydroxypropylflavin, 6,7-dimethyl-9-(3′-hydroxypropyl)isoalloxazine; DL-glyceroflavin, 6,7-dimethyl-9-(1′-DL-glyceryl)isoalloxazine; 4′-hydroxybutylflavin, 6,7-dimethyl-9-(4′-hydroxybutyl)isoalloxazine; D-erythroflavin; 6,7-dimethyl-9-(1′-D-erythrityl)isoalloxazine; 2′-deoxyriboflavin, 6,7-dimethyl-9-(1′-D-2′-deoxyribityl)isoalloxazine; 6′-hydroxyhexylflavin, 6,7-dimethyl-9-(6′-hydroxyhexyl)isoalloxazine; D-alloflavin, 6,7-dimethyl-9-(1′-D-allityl)isoalloxazine. Similarly, the riboflavin-replaced analogs of FMN and FAD are given by prefixing that portion of the trivial name which indicates a change in structure.

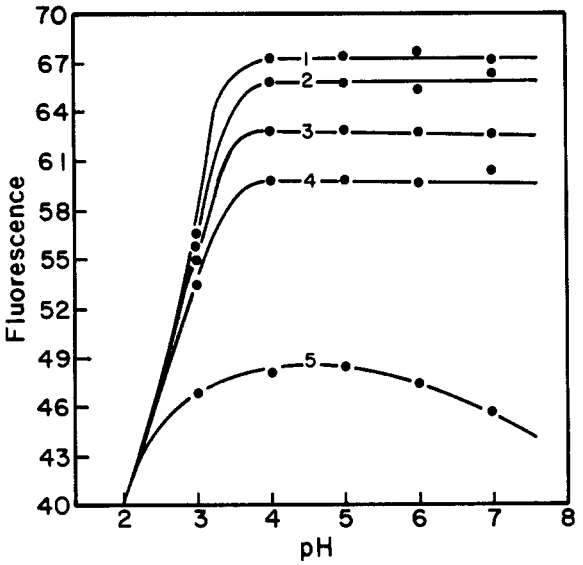

FIGURE 1: Effect of pH change on the intensity of fluorescence of FMN analogs. Curves correspond to: (1) 3′-hydroxypropyl-FMN, 4′-hydroxybutyl-FMN, 5′-hydroxypentyl-FMN, and 6′-hydroxyhexyl-FMN; (2) 2′-deoxyribo-FMN; (3) FMN, DL-glycero-FMN, D-erythro-FMN, and D-allo-FMN; (4) 3-methylribo-FMN; (5), 6,7-dichloro-FMN. Conditions were as given under Methods.

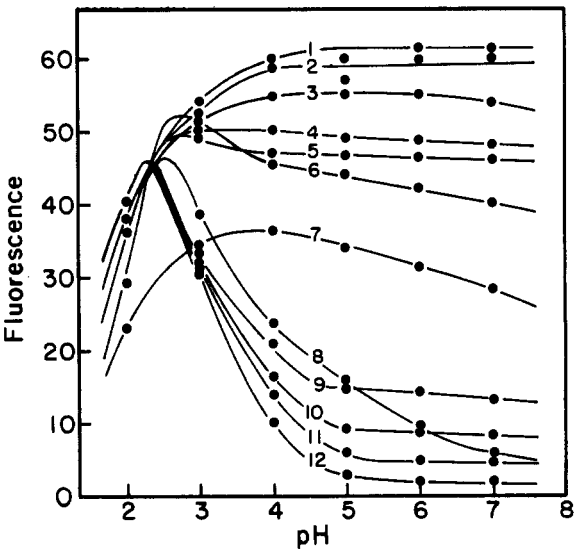

FIGURE 2: Effect of pH change on the intensity of fluorescence of FAD analogs. Curves correspond to: (1) D-allo-FAD; (2) DL-glycero-FAD; (3) FA(1-*N*-oxide)D; (4) 3′-hydroxypropyl-FAD; (5) D-erythro-FAD; (6) 2′-deoxyribo-FAD; (7) 6,7-dichlororibo-FAD; (8) FAD; (9) 3-methylribo-FAD; (10) 6′-hydroxyhexyl-FAD; (11) 5′-hydroxypentyl-FAD; (12) 4′-hydroxybutyl-FAD. Conditions were as given under Methods.

that the dinucleotides were synthesized and appeared relatively pure.

Intensities of fluorescence of FMN and FMN analogs as influenced by changing pH are shown in Figure 1. In the strongly acid range, protonation of the flavins produces nonfluorescent species. From weakly acid to neutral pH, there is a relative constancy in fluorescence of all analogs tested except 6,7-dichloro-FMN which was observed to undergo more rapid photobleaching of fluorescence. This latter analog is considerably less fluorescent than FMN on an equimolar basis. The 3-methylribo-FMN is also somewhat less fluorescent than FMN. Flavin phosphates with glycityl chains, *i.e.*, DL-glyceryl, D-erythrityl, D-ribityl, and D-allityl, exhibit the same fluorescence, while 2′-deoxyribo-FMN is slightly more fluorescent, and analogs with an ω-hydroxyalkyl chain, *i.e.*, 3′-hydroxypropyl, 4′-hydroxybutyl, 5′-hydroxypentyl, and 6′-hydroxyhexyl, are about 10% more fluorescent.

The influence of changing pH on the intensities of fluorescence of FAD and FAD analogs are shown in Figure 2. As with flavins and flavin phosphates, the flavin moieties of these FAD analogs exist as nonfluorescent species under strongly acid conditions. Similar optima for fluorescence intensities near pH 3 are evident with FAD, 3-methylribo-FAD, and the 4′-hydroxybutyl, 5′-hydroxypentyl, and 6′-hydroxyhexyl analogs of FAD. These compounds show quenching of fluorescence at pH values above their optima and exist primarily as internal complexes above pH 5. Little fluorescence quenching attributable to intramolecular complexing occurs with 6,7-dichlororibo-FAD, as the moderately broad decrease in fluorescence at higher pH was also observed with the FMN analog. The 2′-deoxyribo-FAD has an optimum for fluorescence which, though slight, is similar in character to those analogs which are able to form internal complexes. The low magnitude of quenching toward neutral pH is probably largely a reflection of impurity of this preparation. FA(1-*N*-oxide)D does not exhibit significant internal quenching in weak acid to neutral conditions. Lack of such quenching is also apparent with the 3′-hydroxypropyl, DL-glyceryl, D-erythrityl, and D-allityl analogs of FAD.

Delineation of general causes for the inability of certain of the FAD analogs to form intramolecular complexes was investigated further by comparing the magnitude of fluorescence quenching due to intermolecular complexing of AMP or its 1-*N*-oxide with FMN or FMN analogs. AMP quenches the fluorescence of 6,7-dichloro-FMN, though less well than FMN. The fluorescence of FMN is considerably less quenched with AMP 1-*N*-oxide than with AMP. However, AMP quenches the fluorescence of the 3′-hydroxypropyl, DL-glyceryl, D-erythrityl, and D-allityl analogs of FMN approximately as well as FMN itself.

Discussion

It is known that 2′,3′,4′,5′-tetraacetylriboflavin is

more fluorescent than riboflavin or FMN, and consideration has been given to reasons for this phenomenon (Tsibris *et al.*, 1965). The present findings that FMN analogs which lack hydroxyl groups in the side chain at position 9 are more fluorescent than those with glycityl chains afford more direct evidence that some self-quenching in the latter is due to the secondary hydroxyl groups interacting in some manner with the isoalloxazine ring system.

The ability of 4′-hydroxybutyl, 5′-hydroxypentyl, and 6′-hydroxyhexyl analogs of FAD to form intramolecular complexes indicates that hydroxyl groups in the ribityl portion of FAD play no obligatory role in the internal donor–acceptor complexing, and some variation in the length of this connecting portion is allowed. The absence of significant internal quenching of fluorescence with 3′-hydroxypropyl-FAD and DL-glycero-FAD must be due to insufficient length of the three-carbon chains to allow the adenine and isoalloxazine moieties to interact. This property can be seen easily with space-filling molecular models where these particular FAD analogs cannot be manipulated to permit facile overlaying of the necessary regions. Proof that no restriction other than a steric one prevents intramolecular complexing in these cases is proffered by the observation that fluorescence quenching of their FMN analogs occurs by intermolecular complexing with AMP. Though D-erythro-FAD and D-allo-FAD have flavin chain lengths that when extended are similar to their counterparts, 4′-hydroxybutyl-FAD and 6′-hydroxyhexyl-FAD, the folding of the chain which is prerequisite to intramolecular complexing is not so readily accomplished because of steric bulk in molecular models of the former compounds. As expected, their FMN analogs do complex intermolecularly with AMP.

Alkylation of riboflavin at the 3-imino position was originally reported to markedly decrease fluorescence (Kuhn and Boulanger, 1936). Later work (Harbury and Foley, 1958) reversed this conclusion, but no comparison of the fluorescence of equimolar 3-methylribo-FMN to FMN was available. As noted herein, the 3-methyl analog is only slightly less fluorescent than FMN. Methylation at the 3-imino position in the isoalloxazine also has little effect upon the ability of 3-methylribo-FAD to form an intramolecular donor–acceptor complex. The fact that this analog is inactive as a coenzyme for D-amino acid apooxidase (Chassy and McCormick, 1965) cannot be attributed to impairment of an internal complex, the form in which FAD is at least presented to this enzyme.

Although the fluorescence of 6,7-dichloro-FMN is quenched by high concentrations of AMP, little quenching due to internal complex formation is seen with the FAD analog. Since no steric restraints are manifest, charge pertubation may account for these findings. The weaker complexing of FMN by AMP 1-*N*-oxide as contrasted with AMP agrees with the observation of the noncomplexed FA(1-*N*-oxide)D. This is expected, as the adenine ring carbon which bears the 6-amino group in the *N*-oxide is less favorably polarized for charge overlap with the 1-nitrogen of the isoalloxazine. This FAD analog also has little or no activity with D-amino acid oxidase, in which respect it is similar to other adenylate-replaced analogs of FAD (McCormick *et al.*, 1964).

The essential similarity in confluence of the pH optima in the acid region, where maximum fluorescence due to disruption of the internal complexes occurs with all of those isoalloxazine-substituted analogs of FAD which can complex at higher pH, reflects protonation of the adenosine amino with a pK_a near 3.3 (Long, 1961).

Further studies are necessary to allow definitive conclusions to be drawn about the coenzymatic role of the intramolecular donor–acceptor complex of FAD. However, considerably insight now has been gained into factors which contribute to formation of this complex.

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